From the Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

DIETARY NITRATE AND THE MICROBIOTA - MODULATORS OF METABOLIC FUNCTION

Chiara Moretti

Karolinska Institutet

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Dietary Nitrate and the Microbiota – Modulators of Metabolic Function

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By

Chiara Moretti

Principal Supervisor:
Prof. Jon Lundberg
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Pharmacology

Co-supervisor(s):
Prof. Eddie Weitzberg
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Anesthesiology and Intensive Care

Associate Prof. Filip Larsen
Swedish School of Sports and Health Sciences
Department of Physiology, Nutrition and Biomechanics

Opponent:
Associate Prof. Lee D. Roberts
University of Leeds
Leeds Institute of Cardiovascular and Metabolic Medicine
School of Medicine

Examination Board:
Associate Prof. Velmurugesan Arulampalam
Karolinska Institutet
Department of Microbiology Tumor and Cell Biology

Prof. Carl Johan Sundberg
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Physiology

Associate Prof. Susanne Keipert
Stockholm University
Department of Molecular Biosciences
The Wenner-Gren Institute

ABSTRACT

Nitric oxide (NO) deficiency represents a known feature of cardiovascular and metabolic diseases as well as natural aging. The NO metabolites nitrate and nitrite have long been considered as health-threatening food components with potential carcinogenic effects. Surprisingly, more recent research has demonstrated that boosting of a nitrate-nitrite-NO pathway via the diet (mainly green leafy vegetables and beetroot) improves cardiovascular function, mitochondrial efficiency and reduces oxidative stress.

The aim of this thesis was to explore the effects of inorganic nitrate and nitrite with respect to metabolic dysfunction, when this is driven by either unbalanced diets or natural aging. At the same time, we sought to clarify whether the microbiota is an indispensable factor for the bioactivation of dietary nitrate, its cardiometabolic effects as well as the onset of diet-induced obesity.

We demonstrate that inorganic dietary nitrite extends the lifespan of female fruit flies and protects them from age-dependent locomotor decline, thus promoting healthspan. Moreover, nitrite could lower glucose and triglycerides levels in aged female flies. This, together with modulation of dTOR and d*Sir2* gene expression, indicates that nitrite might benefit metabolism during aging by regulating the sensing of nutrients. Furthermore, we show the existence of a nitrite-NO pathway, to which the fly bacteria likely contribute. Similarly, we prove the obligatory role of the host microbiota in bioactivation of dietary nitrate in mammals. In a mouse model of cardio-metabolic dysfunction, we described blood pressure-lowering and anti-diabetic effects as well as protection from hepatic steatosis by dietary nitrate, in the presence of a conventional microbiota. However, when the same disease model was reproduced in germ-free mice, carrying no bacteria, none of these salutary effects of nitrate was achieved. While attributing the cardiometabolic benefits of inorganic nitrate to the host microbiota, in a separate study we show that no such obligatory relationship underlies the general onset of diet-induced obesity. This finding is in stark contrast to the current literature which suggests a causal role of gut bacteria in fat storage.

In conclusion, we here describe previously unknown metabolic effects of dietary nitrate and nitrite which are dependent on the host microbiota. In addition, we show that diet-induced obesity and its complications develop both in the presence and absence of gut bacteria.

LIST OF SCIENTIFIC PAPERS

I. **Dietary nitrite extends lifespan and prevents age-related locomotor decline in the fruit fly**

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Free Radic Biol Med 2020 Sep 24; 160:860-870

II. **The obligatory role of host microbiota in bioactivation of dietary nitrate**

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Free Radic Biol Med 2019 Dec; 145:342-348

III. **AMP-activated protein kinase activation and NADPH oxidase inhibition by inorganic nitrate and nitrite prevent liver steatosis**

Isabel Cordero-Herrera, Mikael Kozyra, Zhengbing Zhuge, Sarah McCann Haworth, Chiara Moretti, Maria Peleli, Mayara Caldeira-Dias, Arghavan Jahandideh, Han Huirong, Josiane de Campos Cruz, Andrei L. Kleschyov, Marcelo F. Montenegro, Magnus Ingelman-Sundberg, Eddie Weitzberg, Jon O. Lundberg and Mattias Carlström

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IV. **Germ-free mice are not protected against diet-induced obesity and metabolic dysfunction**

Chiara H Moretti, Tomas A Schiffer, Xuechen Li, Eddie Weitzberg, Mattias Carlström, Jon O Lundberg

Manuscript

RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

1. **Head-to-head comparison of inorganic nitrate and metformin in a mouse model of cardiometabolic disease**

   Isabel Cordero-Herrera, Drielle D. Guimarães, Chiara Moretti, Zhengbing Zhuge, Huirong Han, Sarah McCann Haworth, Arturo Eduardo Uribe Gonzalez, Daniel C. Andersson, Eddie Weitzberg, Jon O. Lundberg, Mattias Carlström

   Nitric Oxide 2020 Jan 97:48–56

2. **Symbiotic bacteria enhance exercise performance**

   Jon O Lundberg, Chiara Moretti, Nigel Benjamin, Eddie Weitzberg

   Br J Sports Med 2020 April 0;1-2

3. **Microbiota, diet and the generation of nitrogen compounds**

   Mattias Carlström, Chiara H Moretti, Eddie Weitzberg, Jon O Lundberg

   Free Rad Biol Med Oct 2020

CONTENTS

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AO	Aldehyde oxidase
BRJ	Beetroot juice
cGMP	3´- 5´- cyclic guanosine monophosphate
CONV	Conventional
COPD	Chronic obstructive pulmonary disease
CPT1	Carnitine palmitoyl transferase 1
DETA-NONOate	Diethylamine NONOate
DEXA	Dual-emission x-ray densitometry
DR	Dietary restriction
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
GF	Germ-free
GLUT4	Glucose transporter type 4
HbA1c	Glycated haemoglobin
HF	High fat
HNO_2	Nitrous acid
IGF-1	Insulin growth factor 1
IP-GTT	Intraperitoneal glucose tolerance test
IP-IIT	Intraperitoneal insulin tolerance test
L-NAME	Nω-Nitro-L-arginine methyl ester
MAP	Mean arterial pressure
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
ODQ	[1H-[1,2,4]oxadiazolo-[4,3-1]quinoxaline-1-one]

O-GTT	Oral glucose tolerance test
PE	Phenylephrine
RING	Rapid iterative negative geotaxis
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SNP	Sodium nitroprusside
SY	Sugar-yeast
T2D	Type 2 diabetes
VAT	Visceral adipose tissue
WAT	White adipose tissue
WD	Western diet
XOR	Xanthine oxidoreductase
8-pCPT-cGMP	8-(4-Chlorophenylthio)-guanosine 3´, 5´- cyclic monophosphate sodium salt

1 INTRODUCTION

1.1 ENZYMATIC SYNTHESIS, SIGNALING AND BIOLOGICAL FUNCTIONS OF NO

Nitric oxide (NO) is endogenously produced by NO synthases (NOSs). These exist in three isoforms (nNOS, iNOS and eNOS), which catalyse NO production via an oxygen-dependent five-electron-transfer reaction from the amino acid L-arginine. Such reaction requires a number of cofactors and eventually results in equimolar amounts of NO and L-citrulline [1].

Once NO is formed, this reactive free radical gas is rapidly oxidized forming nitrate and nitrite. In blood, NO reacts with oxyhaemoglobin generating nitrate and methaemoglobin, whereas in tissues some nitrite is formed as well [1]. Therefore, plasma levels of nitrate in humans are typically higher than nitrite levels *i.e.* 20-40 µM and 50-300 nM respectively [2,3].

Being one of the most ubiquitous signaling molecules in mammals, NO activates numerous downstream pathways. The most important of these, is perhaps the stimulation of soluble guanylyl cyclase (sGC), leading to production of 3´-5´- cyclic guanosine monophosphate (cGMP) which then activates cGMP-dependent protein kinase (PKG).

This specific signaling pathway is responsible for many of the biologic functions of NO, both at the organ and cellular level. For instance, it mediates relaxation of vascular smooth muscle and platelet aggregation in the cardiovascular system [4,5]. It also triggers angiogenesis and plays an important role in the immune- [6,7] and nervous systems [8,9]. At the cellular level, NO broadly affects mitochondria and their function. Its reversible binding to cytochrome c oxidase is responsible for reduced mitochondrial respiration. At the same time, NO targets complex I of the electron transport chain through S-nitrosation, resulting in reduced generation of mitochondrial reactive oxygen species (ROS) [10–12]. Moreover, by decreasing the expression of mitochondrial proteins that regulate proton leakage, NO also improves mitochondrial efficiency [13].

In 1994, two groups independently discovered that NO formation does not necessarily require NOS enzymes. Instead, salivary nitrite was identified as the source of NO, resulting from nitrite protonation to HNO_2 in the acidic gastric milieu and further decomposition to form NO and other reactive nitrogen oxides [14,15]. Following this line of evidence, systemic NO generation from nitrite was reported soon after in the ischemic heart [16]. In this study as in others [17,18], NO formation from nitrite was once again due to the acidic conditions ($pH < 5$ in the ischemic mouse heart). Subsequent studies demonstrated the existence of several other pathways for nitrite reduction *in vivo*. These involve deoxy-haemoglobin, deoxy-myoglobin, xanthine oxidoreductase, protons as well as reductants such as vitamin C and polyphenols [19]. Interestingly, whereas the enzymatic synthesis of NO is oxygen dependent, nitrite reduction to NO is greatly favoured by hypoxia. As such, we should consider NOS independent NO formation as a backup system that helps maintaining NO homeostasis when the NOS enzymes might not be fully functional [20,21]. In support of this view, the administration of nitrite and its precursor nitrate, possess known therapeutic effects such as in ischemia-reperfusion injury [19,22,23].

1.2 NO GENERATION FROM THE DIET: THE NITRATE-NITRITE-NO PATHWAY

Inorganic nitrate is abundant in green leafy vegetables (spinach, rucola, lettuce) and red beets [24]. It is estimated that a plate of vegetables provides more nitrate than what is formed daily by the combination of all three NOS enzymes [25]. Thus, dietary intake of this anion can be considerable.

When nitrate is ingested, it is rapidly absorbed in the gastrointestinal tract and enters the circulation. Here, its concentration typically peaks after 30 minutes from ingestion and its half-life is about 5-6 hours. While most of the circulating nitrate is eventually excreted via the kidneys, as much as 25% is efficiently taken up by the salivary glands and concentrated in saliva. In the oral cavity, nitrate is reduced to nitrite by commensal anaerobic bacteria [24,26]. Swallowed nitrite enters the acidic gastric environment where it is immediately protonated, forming nitrous acid (HNO_2), which in turn forms NO and other nitrogen oxides [14,15] through a non-enzymatic reaction (so called disproportionation). This process is referred to as "the entero-salivary nitrate circulation" (Figure 1).

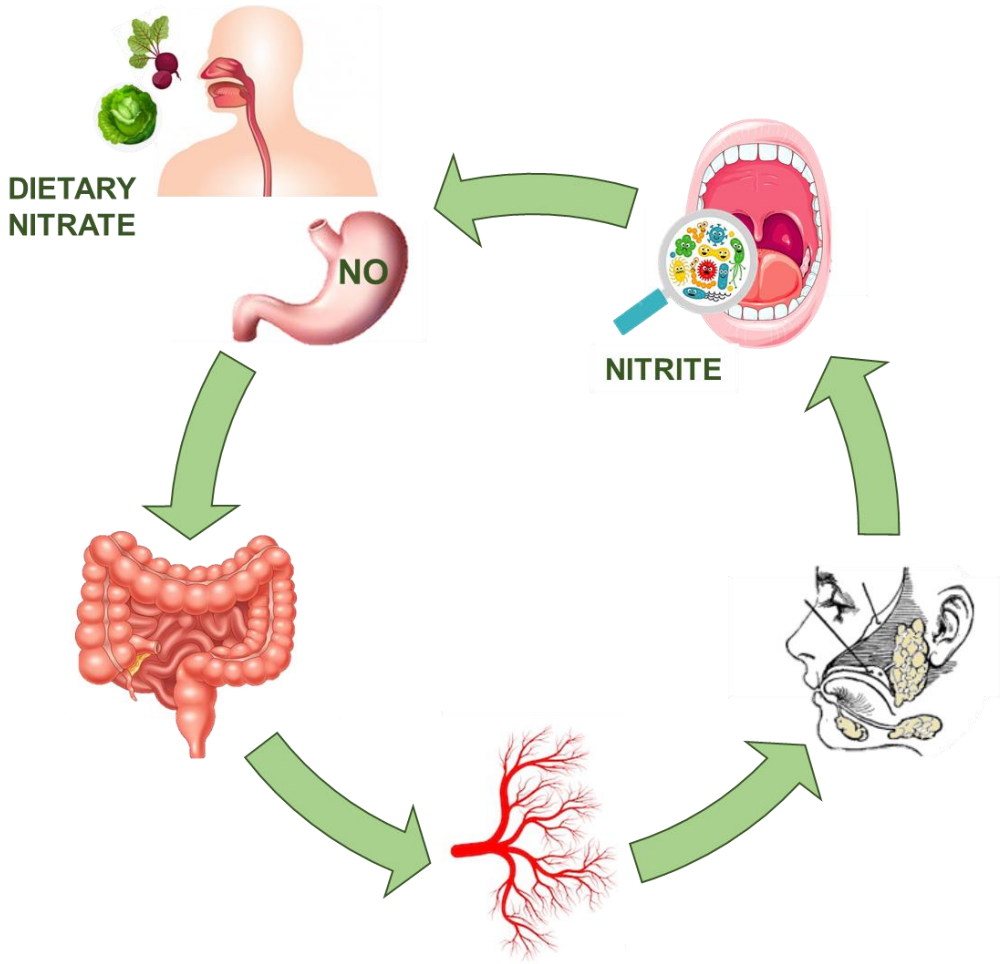

Figure 1. The entero-salivary nitrate circulation.

After a nitrate-rich meal, salivary nitrate levels can approach 10 mM whereas nitrite concentrations are about 10 times lower, ranging from 1 to 2 mM [3]. Thus, salivary levels of nitrate and nitrite are orders of magnitude higher than in normal plasma. This remarkable difference speaks in favour of a pivotal role of the enterosalivary nitrate circulation in preserving NO homeostasis. Therefore, inorganic nitrate supplementation has emerged as a valid alternative source of NO, offering dietary-based opportunities for possible treatment and prevention of some of the most common cardio-metabolic disorders arising from reduced NO synthesis/bioavailability [22].

1.3 BIOACTIVATION OF DIETARY NITRATE

Despite its described salutary effects on blood pressure and metabolism [23,27], inorganic nitrate is likely in itself an inert anion. To achieve the suggested health benefits associated with its intake, a bioactivation process is needed, ultimately forming biologically active NO and other reactive nitrogen oxides. The bioactivation of dietary nitrate starts in the oral cavity, where commensal facultative anaerobic bacteria use nitrate as an alternative electron acceptor to form ATP when oxygen is scarce or to build biomass [26,28,29]. In this process the product is nitrite, an anion much more reactive than nitrate.

In 2004, Lundberg and Govoni described for the first time an elevation in plasma nitrite after nitrate ingestion. Interestingly, disruption of the enterosalivary circulation by avoiding swallowing during a 2 hours period after the nitrate intake, could prevent the plasma nitrite increase in the same individuals [3]. The implication of oral bacteria in this process was established four years later, when the same authors showed that this remarkable elevation in circulating nitrite was nearly abolished by a mouth rinse with an antiseptic mouthwash, immediately before the nitrate load [28]. The physiological importance of bacterial nitrate reduction and its relevance to cardiovascular health was later demonstrated by a number of studies [30–33].

Once nitrate has been bioactivated forming nitrite, a second reduction reaction is needed to generate NO and other nitrogen oxides [34,35]. This final step can also be considered a bioactivation process happening further down in the gastrointestinal tract, specifically in the stomach. As described above, the acidic pH appears to be the main driver in gastric NO formation from nitrite. In fact, when increasing gastric pH with proton pump inhibitors in rats, Pinheiro et al. found attenuation of the blood-pressure reducing effects of nitrite [36]. This observation was recently confirmed in humans [37].

1.4 DIETARY NITRATE AND THE ORAL MICROBIOTA

The relationship between NO and bacteria is complex, yet fascinating. Among its very many biological functions, NO represents an important player in host immune defence. NO production is in fact, one of the major antimicrobial strategies of macrophages [38] and few bacterial strains can survive a heavy NO exposure [39–41]. As an example, nitrite-dependent NO production in the stomach provides protection against potentially ingested enteropathogens [14,15,42]. Although these microorganisms can easily survive at a low pH alone, they cannot survive that same low pH

in the presence of nitrite [42]. Ironically, at the same time, the generation of NO in the stomach requires oral bacteria to produce nitrite from salivary nitrate in the first place. In fact, in germ-free (GF) rats, gastric NO formation is absent even after a nitrate load [43].

Bacterial bioactivation of nitrate is crucial for the physiologic effects and therapeutic potential of nitrate/nitrite-derived NO. One example is the effect of ingested nitrite on gastric mucus thickness and blood flow. In 2004, Björne et al. found that topical application of human nitrite-rich saliva (obtained after ingestion of nitrate) on the rat gastric mucosa, increases mucus thickness and blood flow [44]. The same effect was achieved in rats after 7 days with nitrate addition to drinking water. Clearly these effects of nitrate-derived NO play a physiologic role in maintaining gastric integrity, conferring protection against gastric injury [45]. However, such beneficial effect is lost in a rodent model of stress-induced gastric injury, if the oral flora is depleted [46]. Hence, the obligatory role of oral bacteria in ensuring the gastroprotective effects of nitrate-derived NO. The importance of this bioactivation process is reflected by the clinical complications seen in intubated patients, whose enterosalivary nitrate circulation is disrupted for a prolonged time. In these patients, insufficient levels of gastric NO are thought to contribute to gastric lesions and bacterial overgrowth [47].

Most studies investigating the role of nitrate-reducing oral bacteria, focused on the cardiovascular consequences of disrupting the nitrate-nitrite-NO pathway [28,30–33,48,49]. In a classic study by Petersson and Carlström, rats were pre-treated for 7 days with an antiseptic mouthwash before receiving nitrate in the drinking water for one week. As a result, bacterial depletion effectively blunted the blood pressure-lowering action of nitrate, which was otherwise clear when animals were not treated with a mouthwash [30]. Further, in two separate human studies, an antiseptic mouth-rinse reduced the nitrate to nitrite reduction, affecting blood pressure. These studies suggest that recycling of endogenous nitrate is sufficient to generate enough NO-bioactivity for physiological modulation of blood pressure [31,33]. In this salvage pathway oral bacteria play an important role.

However, mammalian enzymatic routes are also known to contribute to nitrate and nitrite reduction. One example is the mammalian xanthine oxidoreductase (XOR). In 2008, Jansson et al. suggested a role for this enzyme in reducing nitrate to nitrite *in vivo*, under normoxic conditions. Following systemic nitrate administration in GF mice, they noted a marked elevation in plasma nitrite levels that was attenuated by the XOR inhibitor allopurinol [50]. Indeed, this enzyme might play an important role in bioactivation of nitrate under certain conditions (*i.e.* hypoxia) or within specific tissues where it is highly expressed (*i.e.* white adipose tissue). Nonetheless, the contribution of XOR to the nitrate-nitrite-NO pathway still needs to be dissected, especially in humans.

1.5 THE ROLE OF THE GUT MICROBIOTA IN METABOLIC HEALTH AND POSSIBLE INTERACTIONS WITH DIETARY NITRATE

In the past decades, obesity has become a health and social burden to be urgently addressed [51] and researchers around the globe are joining forces to better understand the pathophysiology of this systemic disease, aiming at more successful treatment strategies. To this respect, one major subject of investigation is the gut microbiota.

In 2004, it was observed that GF mice, carrying no bacteria, appear leaner than conventional (CONV) mice with a normal microbiota. Moreover, GF mice colonized with the faecal microbiota of CONV mice, gain weight and show increased fat mass, fasting blood glucose and insulin levels [52]. This was the first evidence of a potential mechanistic involvement of the gut microbiota in energy harvest from the diet and fat storage. In support of these findings, later reports from the same group and others, described protection of GF mice from diet-induced obesity [53–55] as well as an aberrant, obesity-specific gut microbiome [56,57]. These studies triggered intense research efforts investigating host-bacterial interactions in the regulation of metabolism and ultimately weight control. Currently, a key question in the field is whether we should consider the microbiota as a causal factor in the pathophysiology of obesity [58,59].
Supporting the idea of a causal link, faecal microbial transplantation studies have shown that the obese phenotype can be transferred from *ob/ob* mice [56] but also obese individuals [60] to previously healthy GF mice. Nonetheless, clear evidence supporting such hypothesis in humans is still missing [59,61] and some animal studies suggest that the absence of bacteria does not always protect against obesity [62–65].

More recently, we have appreciated the greater metabolic impact of a balanced gut microbial ecology over strain-specific bacterial modifications. Loss of this balance, a state termed "dysbiosis", has been associated with both metabolic [66,67] and cardiovascular dysfunction [68,69]. To this end, one major influencing factor of the gut microbiota is the everyday diet [70].
Generally, diets rich in fiber such as the Mediterranean diet, have been associated with reduced incidence of metabolic diseases [71]. According to recent research, these diets shape the gut microbiota and such changes seem to be involved in their salutary metabolic outcomes (*i.e.* lowered circulating triglycerides and glucose) [72,73].

Animal studies suggest that supplementation with dietary nitrate could be a promising intervention to modulate metabolism and prevent metabolic dysfunction, especially when driven by high fat/high sugar diets [23,74]. It is logical to consider if an interaction could exist between inorganic nitrate (and its metabolites) and the gut microbiota.
Research addressing this question has so far been very limited. On one hand, it is believed that relevant amounts of dietary nitrate would never reach the lower gastrointestinal tract - where most bacteria reside - due to its complete absorption in the upper intestine [75]. In support of this view, studies have shown no changes in faecal microbial structure in rats after nitrate supplementation [76,77]. On the other hand, reduced Firmicutes to Bacteroidetes ratio (opposite to what is known being a sign of dysbiosis) was reported in healthy individuals after three days supplementation with a nitrate-rich fruit and vegetable juice [78]. Thus, indicating potential benefits of dietary nitrate in

preventing gut dysbiosis. Furthermore, very recent research has indicated amelioration of dysbiosis by nitrate in mouse models of colitis [79] and diet-induced obesity [80]. The reverse has also been shown: prolonged consumption (18 months) of a nitrate depleted diet leads to dysbiosis as well as severe metabolic and cardiovascular dysfunction in mice [81].

Thus, a previously unexpected interplay between gut bacteria and the metabolic benefits of nitrate is coming to light. In the future, studies might clarify the specific action of nitrate-rich diets on gut microbial composition as well as the effects of any such modification, with particular respect to metabolic diseases but also lifestyle factors (*i.e.* physical activity, specific dietary regimens, anti-diabetic/obesity medications).

1.6 DIETARY NITRATE AS A MODULATOR OF METABOLIC FUNCTION

A role of NO in maintaining metabolic homeostasis became clear when in 1995, Huang et al. found that mice lacking eNOS do not develop hypertension exclusively, but also metabolic dysfunction with increased abdominal fat, dyslipidaemia and glucose intolerance [82]. In humans, polymorphisms in the eNOS gene have been associated with type 2 diabetes (T2D) [83] and these patients also seem to generate less NO from L-arginine, as compared to healthy subjects [84,85]. Similar effects are seen following chronic NOS inhibition [86]. If NO is such a crucial molecule for maintaining a healthy metabolism, would then nitrate supplementation prevent or restore impaired metabolic function? Carlström et al. addressed this question by supplementing aged $eNOS^{-/-}$ mice with inorganic nitrate at a similar amount to what has been estimated to be produced endogenously by eNOS [87]. After receiving nitrate in the drinking water for 8-10 weeks, the metabolically compromised eNOS deficient mice showed improved glucose tolerance, reduced plasma triglycerides, lower glycated haemoglobin (HbA1c) as well as reduced weight gain accompanied by reduced adiposity. Thus, replenishment of NO bioactivity via dietary nitrate could reverse a metabolic syndrome-like phenotype, supporting the idea of NO as an important modulator of metabolism.

Two years after these findings, Nyström and colleagues found increased pancreatic blood flow and insulin release by inorganic nitrite administered acutely [88], providing further evidence of antidiabetic effects of nitrite-derived NO. In the following years, the antidiabetic effects of stimulating the nitrate-nitrite-NO pathway were studied in various animal models of T2D and metabolic syndrome. In a rat model of fructose-induced T2D, Essawy et al. reported decreased mean arterial blood pressure, improved insulin resistance and decreased adipose tissue index, both when nitrate was administered as a preventive and as a therapeutic intervention, with the greater effects in the first model [89]. Later, another study employed a rat model of T2D induced by streptozocin and nicotinamide. In these animals, the low levels of circulating nitrate and nitrite were restored by nitrate supplementation in the drinking water. At the same time, nitrate improved the animals´ metabolic function (glycemia, lipid profile, glucose tolerance) [90]. Similar results were obtained with chronic oral nitrite administration in a rat model of metabolic syndrome with heart failure [91].

Most importantly, chronic dietary nitrate supplementation has proven effective also when obesity, T2D [92,93] and hepatic steatosis [94] were induced by calorie dense diets. Conversely, wild type mice depleted of nitrate and nitrite from the diet, developed a similar phenotype of that seen in eNOS$^{-/-}$ mice: metabolic syndrome, endothelial dysfunction and even premature cardiac death developing over time (18-22 weeks) [81].
Despite the fact that antidiabetic effects of nitrate/nitrite have been widely described in animal models of obesity and T2D [89–93,95–97], evidence is still lacking in humans. Only one short study was performed in T2D patients, reporting no changes in blood pressure nor improved endothelial function or insulin sensitivity after two weeks beetroot juice (BRJ) intake [98].

The molecular mechanisms behind the salutary metabolic effects of dietary nitrate are progressively being revealed. The insulinotropic effect of nitrite described in the Nyström study, was cGMP dependent [88]. However, some later studies indicate other, cGMP-independent mechanisms.
Glucose transporter type 4 (GLUT4) facilitates the transport of glucose into the cell, representing a crucial regulator of whole body glucose homeostasis [99]. Nitrite has been shown to induce translocation of GLUT4 both *in vitro* [97] and *in vivo* [100], indicating increased glucose uptake by nitrite-generated NO. Similarly, Gheibi et al. found that nitrite supplementation for 8 weeks, stimulates insulin secretion and increases insulin content in the rat pancreatic islets. In the same study, nitrite increased GLUT4 levels in soleus muscle and epididymal adipose tissue in obese, T2D rats [101]. In a separate study, the same group reported similar effects with chronic nitrate supplementation in diabetic rats, together with decreased gluconeogenesis, ROS production and inflammation [93]. Again, Lai et al. showed GLUT4 translocation in skeletal muscle of obese rats following oral administration of inorganic nitrate and nitrite [91]. The proposed mechanisms for GLUT4 translocation are various. In their *in vitro* study, Jiang and colleagues indicated NO derived from nitrite to cause S-nitrosation of GLUT4, thus facilitating its translocation to the membrane of skeletal muscle cells [97]. On the other hand, Lai et al. suggested SIRT3-AMPK activation by long-term nitrite treatment. Activated AMPK would then drive GLUT4 translocation to the plasma membrane of skeletal muscle cells, improving insulin-independent glucose uptake [91].
AMPK is an important cellular energy sensor and its activation is repressed in diet-induced obesity and T2D [102,103]. Thus, restoration of AMPK activity is regarded as a promising approach for the treatment of such diseases [104–107]. Indeed, AMPK activation by dietary nitrate has been reported by several studies using animal models of obesity and diabetes [91,95] as well as in human skeletal muscle fibers from T2D subjects [91]. However, the exact mechanisms of AMPK activation by nitrate/nitrite are debated [108]. One possibility entails a change in cellular energy status triggered by the interaction between the nitrate-derived nitrogen oxide species and the mitochondrial respiratory chain [109].
Newer molecular insights into the metabolic effects of nitrate indicate that short term supplementation with this anion enhances the expression of thermogenic genes and induces browning of white adipose tissue, thus increasing adipocytes oxygen consumption and fatty acid

oxidation *in vivo* [110]. These findings have been recently reproduced in a rat model of T2D following chronic nitrate intake [111]. Of note, browning of adipose tissue has recently emerged as a promising strategy for counteracting obesity and T2D [112,113].

The adipose tissue has gained increasing attention as a metabolic organ greatly involved in insulin sensitivity. As a matter of fact, both the skeletal muscle and the white adipose tissue (WAT) are important sites of glucose disposal. XOR is a known nitrate/nitrite reducing enzyme that is highly expressed in white adipocytes. In a recent report, XOR-mediated nitrate reduction is indicated as a mechanism for the increased WAT glucose uptake and oxidative catabolism in nitrate-supplemented rats [114]. The actions of inorganic nitrate on WAT are worth further research efforts as they might play an important role in the antidiabetic effects of this anion.

At the cellular level, an important player in the metabolic effects of nitrate is the mitochondrion. The interaction between endogenous NO and this organelle is well established [23,108,115]. Already in 1994, nanomolar concentrations of NO were shown to compete with oxygen and reversibly bind cytochrome c oxidase, inhibiting mitochondrial respiration [12] and resulting in reduced mitochondrial ROS production [116]. However, later research has demonstrated that NO and other nitrogen species derived from the nitrate-nitrite-NO pathway, may interact with mitochondria also in other ways [23,108].

In 2011, Larsen and colleagues found reduced mitochondrial proton leak in skeletal muscle biopsies from healthy subject following a 3-day nitrate intervention. This effect observed *in vitro*, was positively correlated with decreased whole-body oxygen consumption during exercise in the same subjects. Ultimately, this study demonstrated that even a short supplementation period with dietary nitrate improves mitochondrial efficiency in humans, increasing the amount of energy produced per oxygen consumed [13]. Accordingly, earlier reports from the same group had shown reduced oxygen cost (VO_2) during exercise, following sodium nitrate intake (0.1 mmol/kg/day for 3 days) in healthy volunteers [117]. Similar effects were reported with BRJ as a source of nitrate (3 days intervention in healthy subjects), proving that dietary sources of nitrate are equally efficacious [118]. Thereafter, the exercise-enhancing abilities of dietary nitrate were reported by a number of studies [13,117–122].

However, a lack of ergogenic effects by nitrate has been described as well [123–125]. The main difference between positive and negative findings being the training status of the subjects. The proposed reasons are various. For instance, consistent training increases muscle oxygenation efficiency, likely limiting nitrite reduction. Moreover, athletes present a higher proportion of type I muscle fibers, which seem to respond less to nitrate supplementation [126]. Nonetheless, circulating nitrate and nitrite appear to be higher in well trained subjects compared to non-athletes [127,128]. This might suggest that a well efficient NOS system is sufficient for optimal NO homeostasis, making nitrate supplementation unnecessary. Similarly, a longer nitrate supplementation via BRJ, while maintaining ergogenic abilities, does not lead to any greater effect if compared to a shorter (3 days) intervention [122].

If nitrate supplementation seems less effective in elite athletes, it might instead represent a valid supplement in situations of reduced mobility due to diseases or the old age. The potential therapeutic benefits of nitrate supplementation will be discussed in the following paragraph.

1.7 DIETARY NITRATE AND METABOLIC DECLINE DURING AGING

In the last two centuries, we have seen life expectancy nearly doubling. However, the length of disease-free lifespan (healthspan) has not been as greatly extended [129], making age-related pathologies some of the most urgent social and economic burdens to be addressed.
Aging represents the major risk factors for cardio-metabolic diseases and it is associated with reduced NO bioavailability and synthesis [130]. Thus, restoration and maintenance of NO homeostasis via nitrate supplementation represents an appealing strategy to address age-related physiological decline.

Following the discovery of the ergogenic abilities of inorganic nitrate, several studies investigated whether nitrate supplementation would improve mobility in older adults. In most of these works subjects were affected by pathologies such as peripheral artery disease [131], obstructive pulmonary disease (COPD) [132–135] or heart failure with preserved ejection fraction [136–138]. Although two of the COPD studies could not detect any effect of BRJ on physical performance [133,139], the overall outcome of these relatively small trials indicates a beneficial role of inorganic nitrate on physical function, together with additional cardiovascular benefits [137].
Similar studies in healthy older individuals are so far scarce. In the first two reports on this type of subjects, nitrate supplementation did not improve functional capacity (walking test, timed up-and-go test and hand-grip test) [140,141] and in a more recent study, no effects of nitrate were detected on exercise tolerance in middle aged-older subjects [142].
Such lack of effect in healthy older adults might suggest a greater relevance of nitrate supplementation when cardio-metabolic function is compromised. Indeed, a similar scenario is portrayed by a number of studies reporting no changes by inorganic nitrate on the blood pressure of healthy or mildly hypertensive older adults [98,140,142–149]. Moreover, aging might lower the ability to bioactivate nitrate, both due to age-related modifications of the oral microbiota [150] or changes in gastric pH [151]. One study supports these hypotheses, in which bypassing the bioactivation of nitrate by intake of nitrite instead, led to improved indices of balance, endurance, and muscle power in older individuals [152].
However, protective effects of nitrate on age-related functional decline have also been described. For instance, when Oliveira and colleagues administered a single dose of beetroot-based nutritional gel to older adults with risk factors for cardiovascular disease, they found increased oxygen extraction and improved oxygen restoration rate during exercise as well as force recovery of handgrip strength [146]. Thus, dietary nitrate seems to ameliorate exercise-recovery time that becomes typically slower with aging. In 2019, a large correlation study compared muscle strength (hand grip test) and physical function (timed up-and-go test) of older women with either

higher or lower dietary nitrate intake (yearly estimation, based on individual semi-quantitative food frequency questionnaire). As a result, higher nitrate intake was associated with enhanced muscle strength and physical function [153]. Yet, another promising study by Coggan et al. found increased maximal knee extensor angular velocity and power in a small cohort of healthy older men and women (65-79 years old), following acute nitrate intake (BRJ) [154]. Although muscle strength is typically considered a good marker for detecting age-related functional deficits, muscle speed and muscle power have been indicated as even more accurate predictors of age-related functional decline [155,156]. Thus, making the findings by Coggan et al. potentially clinically relevant.

As anticipated, aging is accompanied by an overall decline in metabolic function, leading to increased risk for insulin resistance and T2D. In 2011, Sindler et al. showed improved endothelial dysfunction and arterial stiffness by inorganic nitrite in old mice (26-28 months old) [157]. Expanding on this study, Hezel and colleagues explored the effects of nitrate supplementation in age-related hypertension and metabolic dysfunction. In a rat model of natural aging, only two weeks with dietary nitrate supplementation improved glucose tolerance, insulin resistance as well as insulin release [74].
To date, human studies assessing the effects of nitrate on age-related metabolic dysfunction are somewhat lacking. In one preliminary clinical trial by Gilchrist et al., supplementation with BRJ (7.5 mmol nitrate) for two weeks in older, T2D patients did not affect blood pressure nor endothelial function. Similarly, insulin sensitivity was not improved by dietary nitrate [98]. Of note, patients treated with the antidiabetic drug metformin were not excluded from this study. As we now know, both metformin and nitrate are AMPK-activators and this mechanism is likely involved in the glucoregulatory effects of nitrate as well. Thus, nitrate treatment might have failed showing additional effects to those of metformin on glucose homeostasis. This hypothesis is in fact supported by a recent study reporting no synergistic effect of metformin and nitrate on metabolic outputs in a mouse model of diet-induced obesity with hypertension [158].

In summary, more extensive studies are warranted to investigate the potential of dietary nitrate in ameliorating metabolic function in the elderly population. These should explore different doses and length of treatment both with respect to age-related locomotor decline and metabolic dysfunction.

2 AIMS

- To assess the effects of dietary inorganic nitrite on longevity and healthspan in the fruit fly.
- To clarify the importance of the microbiota in bioactivation of nitrate.
- To study the effects of nitrate on dietary induced metabolic dysfunction and the role of commensal bacteria.
- To clarify the overall role of the microbiota in the onset of diet-induced obesity and metabolic dysfunction.

3 MATERIALS AND METHODS

3.1 ANIMAL MODELS

All the *in vivo* studies performed in vertebrates were approved by the Institutional Animal Care and Use Committee in Stockholm and performed in accordance to the guidelines of the National Institutes of Health (NIH), with the EU Directive 2010/63/EU for the conduct of animal research.

Study I

Wildtype *Drosophila melanogaster* (wDah) fruit flies [159] were reared and maintained on a standard sugar-yeast diet (10% SY) prepared accordingly: 10% brewer´s yeast, 5% table sugar, 1.5% agar [160]. Breeding stocks were housed at constant density. Both breeding stocks and experimental flies were housed at constant temperature (25˚C) and humidity (65%), with a 12h:12h light-dark cycle.

Study II

Male C57BL/6J CONV mice were purchased from Janvier Labs (France) at three weeks of age and housed in our animal facilities at Karolinska Institutet. GF C57BL/6J male mice were obtained from the breeding facility at Astrid Fagraeus Laboratory at Karolinska Institutet (Stockholm, Sweden). These mice were housed in gnotobiotic isolators and received sterile food and water until the end of the *in vivo* observational protocol. The germ-free status was confirmed on a weekly basis by culturing faecal samples in aerobic and anaerobic conditions, at +37 °C. The same bacterial cultures were read for up to two weeks. All animals were maintained on a 12 h light/dark cycle, housed at controlled temperature and humidity and given free access to food and water.

From 11 weeks of age, both GF and CONV mice in the treatment groups, were fed a Western diet (40 kcal% from fat, 43 kcal% from carbohydrates, 17 kcal% protein, D12079B, Research diets Inc., New Brunswick NJ), with the NOS inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 1 g/L) in drinking (distilled) water. Together with this, the nitrate treated animals (n=9) received 10 mM of sodium nitrate ($NaNO_3$) while the placebo group (n=6) received 10 mM sodium chloride (NaCl). Control groups of aged-matched GF and CONV mice were kept on a regular rodent diet (n=5; R34, 4% kcal from fat, Lantmannen, Sweden) and tap water. During this 6-weeks treatment period, body weight, food and water consumption were monitored on a weekly basis.

Study III

Male C57BL/6J mice were purchased from Janvier Laboratories at 12 weeks of age and housed in our animal facilities at Karolinska Institutet, at controlled temperature and humidity, with a 12h light/dark cycle. A control group was fed a standard rodent diet (R34, 4% kcal from fat,

Lantmannen, Sweden). The high-fat diet (HFD) and HFD + nitrate groups received a high-fat diet (60 kcal% fat, 20 kcal% carbohydrate, 20 kcal% protein, D12492, Research Diets Inc., New Brunswick, NJ) with the NOS inhibitor L-NAME (1 g/L) in drinking water, with or without inorganic nitrate supplementation (n=6 to 10 per group).
Body weight, food and water consumption were measured throughout the whole 7-weeks study period, after which blood and tissues of interest were collected.
The GF mice (n=17) in this study, received a double irradiated HFD (40 kcal% fat, 43 kcal% carbohydrate, 17 kcal% protein, D12079B, Research Diets Inc., New Brunswick, NJ) and L-NAME (1 g/L) in drinking water for 7 weeks. The germ-free status was confirmed weekly as described in Study II.

Study IV

Aged-matched, male GF (n=10) and CONV (n=6) C57BL/6J mice were obtained from the breeding at Astrid Fagraeus Laboratory, Karolinska Institutet (Stockholm, Sweden) and housed as in Study II. The germ-free status was assessed as described in Study II. All mice in this study received sterile food and water.
From 11 weeks of age, all animals were switched to a double irradiated Western diet containing 40,7% of total calories from fat; 40,6% from carbohydrate; 18,7% from protein (TD96132; Envigo Teklad Diets, Madison, WI) for a period of 16 weeks. Body weight, food and water consumption were monitored weekly. A group of age-matched GF and CONV mice fed a standard rodent diet (R34: 4% of total calories from fat; 9,5% from carbohydrates; 16,5% from protein, Lantmannen, Sweden) is shown for comparison.

3.2 *IN VIVO* STUDIES

Study I

Lifespan assays were performed using aged-matched fruit flies. Briefly, eggs were synchronized and newly emerged flies were allowed a mating period of 48 h. Thereafter, males and females were separated under light CO_2 anaesthesia and 20 flies were transferred to each of 10 experimental vials containing the experimental die (15% SY media prepared as follow: 15% baker´s yeast, 15% table sugar, 1.5% agar). Such diet was supplemented with 0, 0.1, 1, 10 or 100 μM inorganic nitrite ($NaNO_2$), added after cooling the media to 65˚C. Dietary restriction (DR) media consisted of a 5% SY media, prepared as follow: 5% baker´s yeast, 5% table sugar, 1.5% agar. Fresh food was provided every second day. At this time, dead flies were counted. The first day on a dietary treatment is referred as to the first day of adulthood.

A **fecundity assay** was carried out over the first 40 days of the flies´ lifespan as previously described [161]. Aged matched female flies were allocated to each of six independent vials (15 flies/vial) containing the experimental media. Flies were transferred to new food every 3 to 4 days. At this occasion, unhatched eggs were counted using a microscope. The mean number of eggs laid/fly/day was calculated and adjusted for the number of living flies at the time of the

count. During this assay, flies were fed the same media used for the longevity studies and in the presence or absence of inorganic nitrite (0.1 and 1 μM).

Body weight represents the cumulative weight of 10 female flies. Flies were weighed following brief cold anaesthesia and using a precision scale. We report a mean of 7 replicates for each experimental group.

To assess **food intake,** the food tracer method (FD&C BLUE No. 1, Sigma) was chosen and performed as described [161], using aged-matched (14 days old) female flies following a 7 days period on a 15% SY diet with or without the addition of 1 μM nitrite. Results represent 6 replicates of 5 flies each.

Locomotor behaviour was assessed with a rapid iterative negative geotaxis (RING) assay [162] in aged-matched female flies, fed a 15% SY diet with or without 1 μM nitrite and kept in the same conditions as during the longevity experiments. The RING assay was repeated 7, 30 and 44 days after allocation of the flies to the experimental media. The six tubes composing the RING apparatus contained 15 to 20 flies belonging to randomly assigned treatment groups. Flies were transferred into these tubes without anaesthesia. Flies were knocked down to the bottom surface of the tubes by firmly tapping the apparatus three times on the surface of the bench. A picture was taken after three seconds from the last tap of the apparatus with a digital camera located 1 m from the RING apparatus. The procedure was repeated after 1 min recovery time. Seven pictures were taken for each set of the experiment. Six independent vials (n = 6) were tested per each treatment group. The height climbed by the flies was scored from every and each of the 7 pictures taken, in a blinded fashion. Finally, the mean value of the height was calculated.

Study II

All the *in vivo* procedures were performed within the same day to avoid contamination of the GF mice. The same identical protocol was used for CONV mice.

Blood pressure recordings were performed by non-invasive tail cuff technique (Kent Scientific, Torrington; CT, US). After a 4 h fasting period, **fasting glycemia** was measured by sampling blood from the tail vein and using a portable glucose-meter (FreeStyle Lite; Abbott Diabetes Care Inc). Thereafter, an **intraperitoneal glucose tolerance test** (IP-GTT) was performed by injection of a D-glucose solution (2 g/kg of body weight) followed by blood glucose measurement (as described above) at 15, 30, 60 and 120 min from administration of the glucose dose. Mice were then anaesthetized (light isoflurane-anaesthesia - Forene; Abbott Scandinavia AB, Solna, Sweden) in order to assess the **body composition** by dual-emission x-ray absorptiometry (DEXA; Lunar PIXImus densitometer; GE Medical-Lunar, Madison, WI, USA). Finally, blood and tissues of interested were collected and processed according to planned future analyses.

A separate group of GF male (Fv/BN; n = 7) and CONV (C57BL/6J; n = 14) mice received L-NAME (1 g/L) in the drinking water for 3-7 days. Following a 5 h fasting period, baseline blood pressure was measured over the next hour, as described above. After this procedure, mice were orally administered a solution of sodium nitrite ($NaNO_2$, 15 mg/kg of body weight; pH=7) or vehicle (control). The maximum volume of liquid administered was set at 1% of the animal´s body weight. The nitrite dose was chosen upon consideration of the current literature, indicating the effectiveness of such dose in lowering blood pressure in rodents when administered orally [163,164]. Blood pressure was measured 90 min following nitrite administration.

Study III

An **intraperitoneal glucose tolerance test** (IP-GTT) was performed as described in Study II, 5 weeks after the start of the dietary intervention.

An **intraperitoneal insulin tolerance test** (IP-ITT) was performed 6 weeks following the start of the HFD challenge. After measurement of fasting blood glucose, mice were injected with insulin (0.75 IU/kg of body weight; Novorapid 100 IU/ml, Novo Nordisk A/S, Bagsvaerd, Denmark). Glucose levels were then measured from a blood sample taken from the tail vein at 15, 30, 60 and 120 min after the insulin injection. For this procedure, animals were not fasted.

Blood pressure and **body composition** were assessed after 6 weeks dietary treatment as described in Study II. In this study, mice underwent a training period allowing them to adapt to the tail cuff equipment. Following this procedure, blood pressure was recorded daily for three days. The average of the daily measurements for each mouse was used for statistical analyses.

Study IV

Fasting glycemia was measured as described in Study II, following 6 h fasting and after 16 weeks of dietary challenge.

An **oral glucose tolerance test** (O-GTT) was performed in this study, by administration of a 20% D-Glucose solution (in saline) giving a final dose of 2 g/kg of body weight. Glycemia was measured as described above at 15, 30, 60 and 120 min after the glucose dose. Immediately after, the animals were anaesthetized and **body composition** was assessed by DEXA, as described in Study II. Thereafter, blood and organs of interest were collected, processed according to future analyses and snap frozen. Visceral adipose tissue (VAT) and liver were weighted fresh while gastrocnemius muscles were dissected and kept in an isolation media on ice and at 4˚C over-night before mitochondria were isolated.

3.3 *EX-VIVO* EVALUATION OF VASCULAR REACTIVITY

Study II

Mesenteric arteries were collected immediately after sacrifice and kept in ice-cold Krebs solution. Arterial rings (1-2 mm) were obtained from the third branch of the arteries and mounted in myograph chambers (model 620 M, Danish Myo Technology, Aarhus, Denmark), continuously perfused with oxygenated Krebs solution, as previously described [165]. Following stabilization and washout protocols (described in [33]), phenylephrine (PE; 1 μmol/L) was used to pre-constrict the arterial rings, thus obtaining a basal tone of approximately 50% of its diameter.

To investigate endothelium independent NO vasorelaxation, cumulative doses of sodium nitroprusside (SNP; 10−9 to 10−4 mol/L) were added to the chambers at steady-state plateau.

The vascular responses are presented as % relaxation from the PE pre-constricted vessels. EC50 values were calculated using GraphPad Prism v. 8.0.

3.4 *IN VITRO* STUDIES

3.4.1 Cellular studies

Study III

Human hepatic cell line HepG2 was used to test the effects of nitrite on metabolically induced hepatic steatosis *in vitro*. DMEM medium (5.5 mM glucose) with 10% fetal bovine serum, penicillin and streptomycin (50 mg/L) was used to allow cell growth. Cells were maintained at 37 °C and 5% CO_2. A model of steatosis was created by incubation of the cells (serum-free culture medium) with 25 mM glucose, 10 nM insulin and 240 μM FFA (Palmitic and Oleic acid mixture 1:1). When indicated, 10 μM sodium nitrite ($NaNO_2$) was added for 24, 48 or 72 hours. When pharmacological inhibitors were used (Febuxostat 50 nM; Raloxifene 50 nM; ODQ 10 μM; Compound C 20 μM), these were added 30 min before the 24 h treatment of the cells. Instead, Tempol (100 μM), NOX2/4 inhibitor (GLX481304, 1-50 μM), GLX7013114 (0.2-2 μM), the slow releasing NO-donor DETA-NONOate (5 μM) and cGMP analogue 8-pCPT-cGMP (10 μM) were administrated together with the steatosis-inducing mixture. Tryphan Blue exclusion assay and PrestoBlue® cell viability assay were used to verify cell viability.

3.4.2 Cellular, biological and biochemical analyses

Study I

The **gene expression** of dTOR and *dSir2* was determined in female and male flies kept on a 15% SY diet with or without the addition of 1 μM nitrite, since 48 h after hatching. After 30 days, flies were collected under light CO_2 anaesthesia and snap frozen. Homogenates for mRNA extraction were obtained from 3 whole flies. mRNA was extracted using Trizol reagent

(ThermoFisher), according to the manufacturer´s instructions. Expression of the genes of interest was performed using SYBR Green (Applied Biosystems) real-time quantitative PCR (7500 Fast Real-Time PCR System, Applied Biosystems). Primers used are: d*Sir2*-F: 5′-ATGGATAAGGTTCGACGCTTCT-3′ d*Sir2*-R: 5′-AGCCGTCAAAACTCAAATCTGG-3′; dTOR-F: 3' – GGCCGTCCAGGTTCAAAAAC - 5'; dTOR-R: 3' – AATCCGGCGATAGTTCCGTC – 5'. Gene expression was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method, relative to the level of Alpha-tubulin84B: Alpha-tubulin84B-F 5′-GCTGTTCCACCCCGAGCAGCTGATC-3′; Alpha-tubulin84B-R: 5′-GGCGAACTCCAGCTTGGACTTCTTGC-3′. Each sample was assessed in triplicates and 6-8 biological samples per each treatment group were assayed.

Mitochondrial function and hydrogen peroxide (H_2O_2) production were studied in 30 days old female flies, raised as above (paragraph 3.2) and fed a 15% SY diet with or without 1 µM sodium nitrite ($NaNO_2$). Thorax muscle fibers were obtained following dissection of the fly thorax and permeabilized as previously described [166]. Three dissections were kept in 1 ml of biopsy preservation solution (BIOPS) and kept on ice until the end of the dissection process. Dissected fibers were incubated in 1 ml saponin solution (final concentration 50 µg/ml; Sigma S 2149) for 20 minutes (permeabilization) and then transferred to 1 ml incubation media (MIR05) for 10 min and let re-equilibrate, on ice and with mild agitation. After the re-equilibration step, dissections were dried on filter paper and wet weight was assessed with a microscale (0.01 mg resolution).

Mitochondrial oxygen consumption and hydrogen peroxide production were measured simultaneously at 37 °C by high-resolution respirometry (Oxygraph-O2K, Oroboros Instruments, Innsbruck, Austria). For calibration, horse radish peroxidase (HRP) and Amplex UltraRed, 18 µM standard were added to MIR05, in the presence of 10 mM pyruvate and 2 mM malate. Once reached a stable background Amplex Ultrared autooxidation, permeabilized muscle fibers were added to each chamber (containing 2 mL MIR05). Proton leak dependent respiration (LEAK) was measured at this stage. 5 mM ADP was added to measure maximal complex I dependent (state 3 PM) followed by 20 mM succinate (state 3 PMS) to detect complex I+II dependent oxidative phosphorylation capacity (OXPHOS). 0.5 µM rotenone was added to measure complex II dependent OXPHOS (state 3 S). Finally, 2.5 µM antimycin was used to detect non-mitochondrial oxygen consumption and 0.5 mM TMPD + 2 mM ascorbate were added for detection of Complex IV activity.
H_2O_2 production was subtracted from the background and respiratory control ratio (RCR) is represented by the ratio 3PM/LEAK respiration. Media and solutions were prepared as previously described [167]. Data presented were adjusted for autoxidation of TMPD ascorbate at the relevant oxygen tension and normalized with each sample´s wet weight. A total of 9 samples (3 dissections each) were studied.

Total triglycerides (TAG) and glucose levels were assessed in homogenates prepared as described [168] and using five 30 days old female flies. Insects were fed a 15% SY diet with or without 1 μM nitrite since the first day of adulthood.
To quantify TAG levels, homogenates were prepared in ice-cold PBST (PBS + 0.05% Tween-20) using a pellet-pestle, heat-treated and incubated with either PBST for free glycerol measurement or triglyceride reagent to digest TAG and detect the glycerol backbone. Absorbance was measured at 540 nm using a spectrophotometer. Finally, TAG concentration was calculated: *[free glycerol] - [total glycerol]*. Each sample´s TAG content was determined based on a triolein-equivalent standard curve.

Total glucose was measured with a Hexokinase (HK) assay. Flies were fasted for 6 h before being homogenized in ice-cold PBS as above. The obtained samples were heat-treated and 100 μl of HK reagent (Sigma; GAHK20) were added to 30 μl of sample. Absorbance was measured at 340 nm and free glucose concentration determined by comparison with a glucose standard curve.
For both TAG and glucose assays, samples were plated in duplicates on a 96 well plate. The final concentrations presented here have been normalized to the total amount of protein/sample which was quantified by Bradford assay.

Study III

Tissue **NOX activity** was detected by a lucigenin-dependent chemiluminescence assay (AutoLumat LB953 Multi-Tube Luminometer; Berthold Technologies, Bad Wildbad, Germany). Results were normalized to protein levels.

Superoxide production in HepG2 cells was detected by injecting the substrate NADPH (100 μmol/L) in the presence of dark-adapted lucigenin (5 μmol/L) to then measure the chemiluminescence signal. **NOX-derived superoxide and hydrogen peroxide in liver tissue** was performed by measuring Amplex Red-derived fluorescence in tissue homogenates. Fluorescence was measured for 120 min (excitation 530 nm; fluorescence detection 590 nm). The signal was normalized according to the protein concentration and presented as % changes of the respective control animals.
NADPH-derived ROS detected by lucigenin-chemiluminescence technique in HepG2 cells was validated using superoxide dismutase (SOD) and catalase to show that the latter did not influence the signal while SOD almost completely abolished it, thus demonstrating that this assay is predominantly assessing superoxide and not hydrogen peroxide production.

Superoxide and NO species were detected by **Electron Paramagnetic Resonance (EPR).**

Neutral lipid accumulation in cells and liver was detected by Oil Red O (ORO) staining. Fresh liver tissue was embedded in OCT cryomount embedding medium (Histolab Products AB, Sweden) and snap frozen. The staining procedure was performed on frozen sections (10 μm). Briefly, after fixation with 1% paraformaldehyde (10 min at 4˚C), sections were rinsed

and let air dry for a few minutes. Absolute propylene glycol was added for 5 minutes before a 10 min incubation with pre-warmed Oil Red O solution (0.5% in absolute propylene glycol) and followed by differentiation in 85% propylene glycol for 3 min. After rinsing, Mayer´s Hematoxylin was added on each section for 30 sec. Sections were finally washed and mounted with glycergel mounting medium before acquisition of images. Lipid deposition was quantified from the images in a blinded fashion (ImageJ software).

Hepatic triglyceride content was assessed by Triglyceride Assay Kit (Abcam, ab65336)**.** according to the manufacturer´s instructions.

mRNA expression levels of the genes of interest were determined by real-time PCR in liver homogenates, using Power SYBR Green Master mix (Thermo Fisher Scientific). RNA extraction was performed with a RNeasy Mini Kit (Qiagen, Sollentuna, Sweden). Relative changes in mRNA expression were calculated using the ΔΔCt-method and normalizing the results to the housekeeping gene β-actin. Primers sequences are found in Study III supplementary information.

Western blot analyses were performed to detect proteins of interest in HepG2 cells and liver tissue. To do so, cells were lysate in the presence of protease inhibitor cocktail and Serine/Threonine Protein Phosphatases or Tyrosine Protein Phosphatases inhibitors. Liver homogenates were obtained using a bullet blender device and zirconium oxide beads (0.5 mm). Polyvinylidene difluoride membranes were washed with Western Blot Stripping Buffer before incubation with the anti-phospho protein primary antibody, to eventually detect the phosphorylated protein. Vinculin was used as loading control. For details see Study III supplementary information.

Study IV

Hepatic triglycerides were quantified using a Triglyceride Colorimetric Assay kit (Cayman Chemical Company).

Lipid accumulation in hepatic tissue was assessed by Oil Red O staining of 10 µm-thick liver sections, as described in Study III.

CPT1 activity was studied in isolated mitochondria from gastrocnemius muscles. These were kept in isolation media (sucrose 250mM, HEPES 10mM, EGTA 1mM, BSA 1g/L, pH 7,4) on ice at 4˚C over-night. On the following day, samples were weighted and homogenized using a potter elvehjem homogenizer on ice in presence of proteinase (0.2 mg/ml). Homogenates were resuspended in 3 ml isolation medium and centrifuged (700 × g for 10 min). The resulting supernatant was further centrifuged (10,000 × g for 10 min). Pellets were washed to remove the buffy coat and recentrifuged (7000 × g for 5 min). Finally, they were washed and diluted (0.3 µl mg^{-1} initial tissue weight) in preservation medium (EGTA, 0.5 mM; MgCl2, 3 mM; K-lactobionate, 60 mM; taurine, 20 mM; KH2PO4, 10 mM; HEPES, 20 mM; sucrose, 110 mM;

histidine, 20 mM; vitamin E succinate, 20 mM; glutathione, 3 μM; leupeptin, 1 μM; glutamate, 2 μM; malate, 2 μM; BSA 1 g/l and Mg-ATP 2mM) and kept on ice until analysed.
CPT1 activity was studied with high resolution respirometry (O2-K, Oroboros, Austria) and mitochondrial respiration measured in the presence of palmitoyl-CoA (40μM), I'carnitine (0.5mM), malate (0.1 mM) and ADP (2.5mM). DatLab7 (Oroboros) was used for data acquisition. Respiration was normalized to the mitochondrial protein.

3.4.3 Nitrate, nitrite and NO detection

Study I

NO generated by *Drosophila* microbiota was assessed in bacterial cultures obtained by homogenizing fruit flies in Mueller Hinton bacterial culture media, using a sterile pestle. Bacteria were grown anaerobically overnight, in an air-tight chamber together with an anaerobic pouch (AnaeroGen compact AN0035, Oxoid Basingstoke, England) at 37˚C and with mild shaking. NO was detected in air samples obtained from three gas tight infusion bags, made of a multilayer double wound film (M312), after filling them with nitrogen gas (500 ml) and the bacterial suspension + 100 μl of a sterile sodium nitrite solution ($NaNO_2$; 1 mM final concentration) or sterile Mueller Hinton (control). NO gas concentrations were measured with a rapid response chemiluminescence system (Aerocrine AB, Stockholm, Sweden). Air was sampled every 2, 4, 6 and 8 hours. Bags were otherwise kept at 37˚C, with continuous mild shaking. This procedure was repeated in two independent experiments. The instrument's detection limit for NO was 1 ppb. At the end of the experiment, the pH of each bag´s content was assessed with a tritest pH revealing paper.

Nitrite consumption was determined in flies´ homogenates, prepared as described above. Penicillin-Streptomycin (10,000 Units/ml Penicillin, 1000 μg/mL Streptomycin; Gibco) was added (100 μg/ml final concentration) to two aliquots and the resulting samples were incubated for 1 h in anaerobic conditions at 37˚C. At this stage, two new aliquots were sampled from the whole homogenate and nitrite (1 mM) was added to half of all aliquots. Samples were maintained at 37˚C in anaerobic conditions for the entire duration of the experiment. Immediately after nitrite addition (time 0) and every 2 h up to 8 and 24 h, 50 μl of sample were extracted. An equal volume of methanol (Methanol for HPLC, ≥ 99,9%; Sigma-Aldrich) was added and samples were stored at -20 ˚C until analysis. For nitrite detection, samples were centrifuged (10000 g x 10 min at 4 ˚C) and injected into a HPLC system (ENO-20) with a Hamilton syringe. Nitrate and nitrite were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. Then, nitrite was derivatized with Greiss reagent to form diazo compounds which were detected at 540 nm.

DAF-FM DA was detected in whole fly homogenates prepared from 5 untreated female flies for each sample (n=6). Homogenates were spun-centrifuged at 4˚C, to precipitate any debris and supernatants were plated in duplicate on a black, clear bottom 96 well plate (Costar, 3603),

with or without 1 μM sodium nitrite ($NaNO_2$) and the fluorescent probe DAF-FM DA (5 μM). Fluorescence was measured with a microplate reader (SpectraMax iD3, Molecular Devices) every 5 min for 1 h at 28 °C, with excitation 465 nm and emission 515 nm. The average fluorescence values for each duplicate were normalized to the protein content of each sample, obtained by Bradford assay.

Studies II and III

Plasma and tissue nitrate and nitrite were analysed by HPLC (ENO-20) as described previously [87,169] and in Study I. In these studies, 10 μl of plasma were used.

3.5 STATISTICAL ANALYSES

In all studies data are presented as mean ± SEM, unless otherwise stated. A *t*-test or Mann-Whitney test were used for comparisons between two groups and one-way or two-way ANOVA for comparisons among two or more groups. The appropriate post-tests were used for multiple comparisons.

In Study I, a log-rank (Mantel-Cox) test was used for comparisons between two survival curves.

In Study II, delta mean arterial blood pressure (MAP) was calculated by subtraction of MAP absolute values (mmHg) at baseline and after oral administration of vehicle or nitrite. Comparison of control and nitrite treated animals, within each group of animals (CONV and GF) was carried out using a parametric unpaired t-test.

A *p*-value of less than 0.05 was considered statistically significant and statistical analyses were performed using GraphPad Prism, version 7 (Study III) or version 8 (GraphPad Software).

4 RESULTS AND DISCUSSION

4.1 STUDY I

Dietary nitrite extends lifespan and prevents age-related locomotor decline in the fruit fly

4.1.1 Inorganic dietary nitrite extends lifespan in the fruit fly

Based on existing evidence on the salutary cardiovascular, metabolic and antioxidant effects of dietary nitrate, we addressed the question whether an attempt to stimulate NO production via the diet, would preserve health during natural aging, thus extending longevity.

To do so, we based our investigations on the short-lived model organism *Drosophila melanogaster*. Longevity studies were performed in both female and male fruit flies (Figure 2E; F), fed a 15% sugar-yeast (SY) media with the addition of 0, 0.1, 1, 10 and 100 μM sodium nitrite ($NaNO_2$). In our survival studies, the lowest nitrite concentrations, 0.1 and 1 μM, significantly extended the median lifespan of female flies by 9 and 15%, respectively (Figure

2A; B). At higher nitrite concentrations (10 and 100 µM), median lifespan extension (by 11 and 6%, respectively) was not reflected by a statistically significant increase in cumulative survival (Figure 2C; D). Nitrite did not affect lifespan in male flies (Figure 2F).

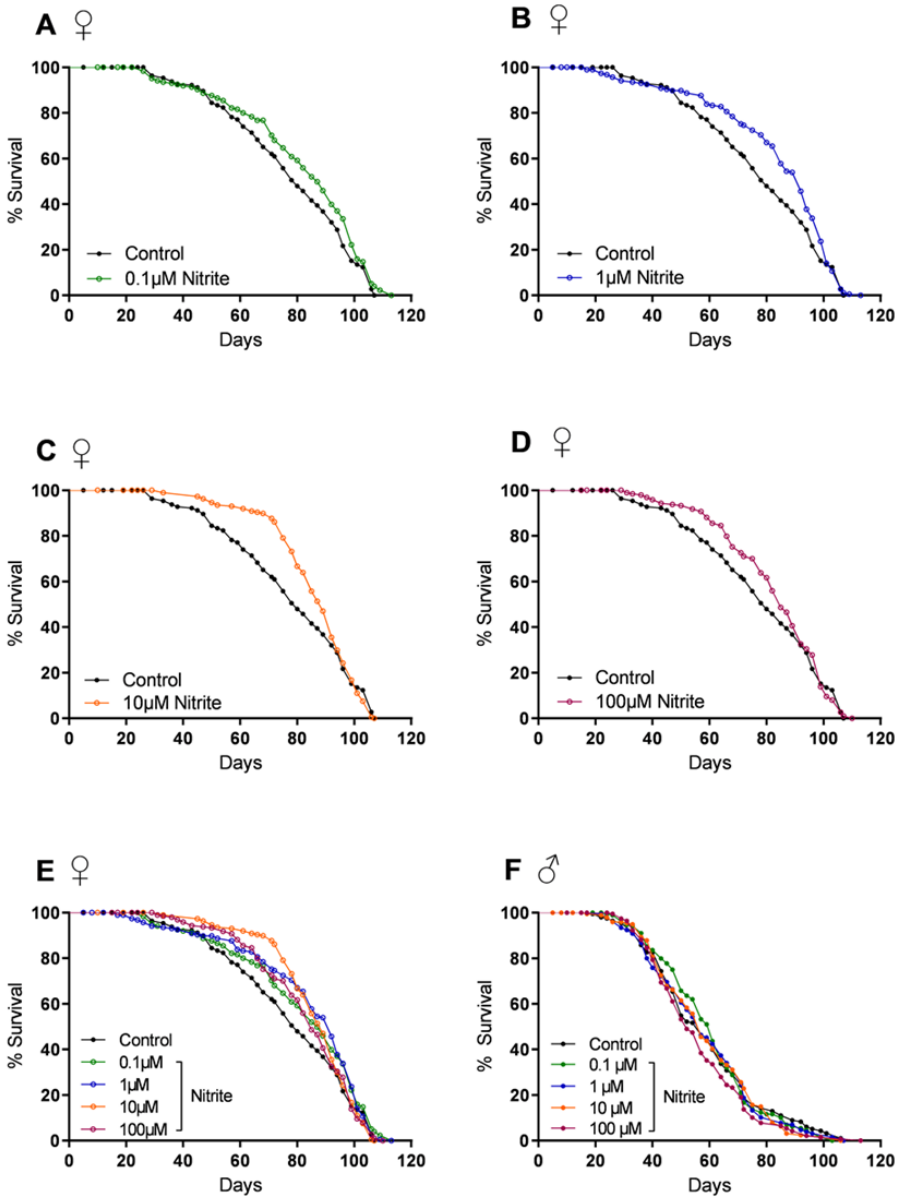

Figure 2. Survival curves of female flies fed a 15% SY media with 0.1 µM (A; n=199; $\chi 2$=5.268; p=0.022); 1 µM (B; n=197; $\chi 2$=5.326; p=0.021); 10 µM (C; n=197; ns) and 100 µM inorganic nitrite (D; n=202; ns), were compared to untreated flies (control; n=207). Log-rank test was used for comparison between two curves. Reported *p*-values indicate comparisons nitrite vs control. Cumulative survival of female (E) and male (F) flies.

To our knowledge, a nitrate-nitrite-NO pathway has never been described in fruit flies. As nitrate would need bacterial activation in order to form NO [3,19,170], we decided to bypass this reduction step by studying the effects of nitrite rather than nitrate in the fly.

The fact that only the lowest concentrations of dietary nitrite significantly extended the fly lifespan might seem surprising. Nevertheless, a U-shaped response to nitrite has been reported previously in animal models of hepatic and myocardial ischemia-reperfusion injury, whereby nitrite was effective at low but not at high concentrations [171].

In our study, nitrite extended lifespan in female but not in male flies. Notably, other dietary interventions (*i.e.* dietary restriction) [172,173] and molecules (resveratrol, rapamycin) that extend longevity are often more effective in females [174,175]. The reasons for sex differences in longevity are so far speculative, both in the case of intervention-based manipulations of the lifespan but also as a result of physiologic aging [176]. Two major driving factors have been proposed: sex differences in energy demand and allocation and sex differences in the insulin/IGF-1 signaling [172]. In *Drosophila*, energy expenditure and intake seem to differ between males and females [177] and also in mammals, studies have indicated higher energy demand in females [178,179]. It is possible that females across species generally require higher energy intake for reproduction. This might even be amplified in flies. In support of this idea, *Drosophila* female´s lifespan is shortened by mating [180–182].

Alternatively, the described sex dimorphism in the insulin/IGF-1 pathway could explain sex differences in longevity. This hypothesis arouse from evidence that downregulation of this pathway leads to greater lifespan extension in females than in males [183–185].
Other explanations have been suggested, based on important physiologic differences between sexes. For instance, the antioxidant and anti-inflammatory properties of oestrogens have been proposed as protective of the female lifespan in mammals [176] and resveratrol, a phytoestrogen, shows greater pro-longevity effects in females [186].

Although median lifespan was significantly increased by 0.1 and 1 µM nitrite, maximum lifespan was left unaffected. One possible explanation is that the extension in median lifespan produced by dietary nitrite might be secondary to improved healthspan (defined as the length of life free from disease). Demographic studies in *Drosophila* have delineated two possible reasons for lifespan extension: either decreased rate of aging (*i.e.* cold exposure in flies) [187] or reduced likelihood of death resulting from accumulation of age-related damage [188]. The latter seems to be the case of dietary restriction (DR) [187] which, similarly to nitrite, affects median lifespan more than maximum lifespan [172].

To verify whether the effects of nitrite would be secondary to a DR effect (*i.e.* by alteration of food palatability leading to reduced food intake) we measured the body weight as well as food intake and egg laying in female flies. Body weight and food consumption were not altered by nitrite, confirming a “true” nitrite-driven effect (Figure 3B; C). Also, female fecundity was unchanged in flies receiving nitrite as compared to controls (Figure 3A).

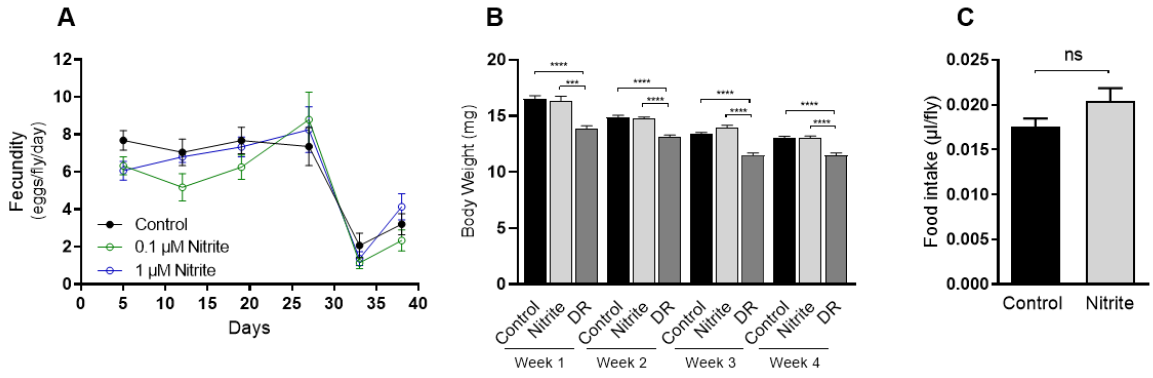

Figure 3. Fecundity assay (A), body weight (B) and food intake (C) of female flies fed a 15% SY diet with or without (control) nitrite supplementation (0.1 and 1 µM). Data are shown as mean ± SEM. *p*-values were determined with a two-way ANOVA for egg-laying (n=6 per group). Body weight (n= 8-7 per group) was analysed with one-way ANOVA to compare treatment groups within the same time-point. Food intake (n=6 per group) was analysed by *t*-test.

Although increased longevity is often paired with decreased fecundity [189], studies are indicating that this dogma of aging biology might need reconsideration. For instance, both resveratrol and a selective TOR inhibitor could increase the fly lifespan without affecting fecundity [190–192].

4.1.2 NO generation from nitrite in Drosophila

One important question to address is whether fruit flies can reduce nitrite to biologically active NO. Knowing that anaerobic gut bacteria, at least in mammals, are able to carry out such reduction reaction [193], we produced anaerobic bacterial cultures from whole fly homogenates to then measure NO gas formation from inorganic nitrite. Following inoculation with 1 mM nitrite, anaerobic fly bacteria immediately produced large amounts of NO gas (Figure 4A). In the same experimental setting, we strengthen this evidence by measuring also bacterial nitrite consumption instead of NO production exclusively. As expected, nitrite concentrations declined steeply 6 hours after nitrite addition to the bacterial cultures, becoming completely undetectable after 8 hours. On the contrary, when bacteria were eliminated using an antibiotic mix, nitrite concentrations remained stable over 24 hours (Figure 4B). Furthermore, at the end of these experiments, we noted that bacterial cultures had become acidic (pH 4-5) as compared to their control (pH 7-8). Although this evidence speaks in favour of a predominant role of bacteria in "bioactivating" nitrite in flies, we wanted to test the hypothesis of a potential non-bacterial enzymatic contribution. Therefore, we measured NO-related species in whole fly homogenates in the presence and absence of nitrite (1 µM) and in aerobic conditions. Although the assay we used does not directly detect NO, DAF-FM DA remains a sensitive fluorescent probe of common use for assessing NO formation in biologic samples. In this experiment, nitrite addition increased the fluorescent signal in whole fly homogenates (Figure 4C). This experiment confirms that fruit flies are able of NO generation from nitrite and we speculate a contribution from host nitrite-reducing enzymes.

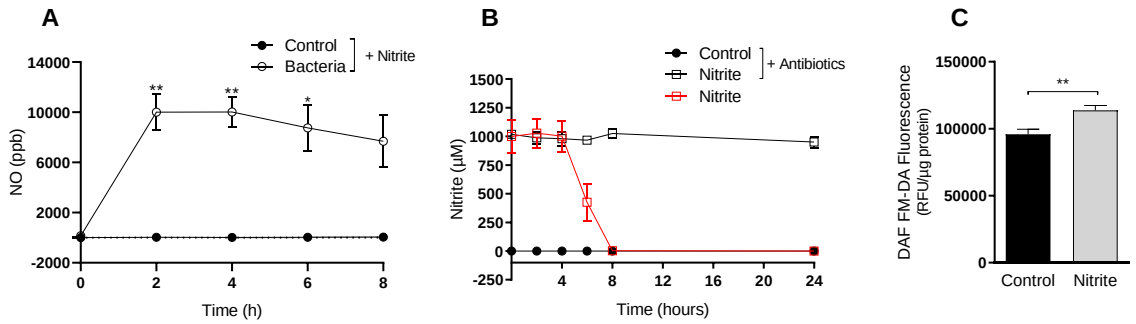

Figure 4. NO gas generation (ppb) by anaerobic bacterial cultures from flies after inoculation with 1 mM $NaNO_2$ (n=3 in two independent experiments; A). Nitrite consumption by *Drosophila* anaerobic bacteria, in the absence (control) or presence of 1 mM nitrite and with or without antibiotics (Penicillin-Streptomycin, 100 mg/ml; B). Detection by DAF-FM DA of NO-related species derived from 1 µM nitrite in whole fly homogenates during aerobic conditions (C; n=6). Data are shown as mean ± SEM. Statistical significance was determined by two-way ANOVA with Tukey correction for multiple comparisons (A; B) and *t*-test (C).

Overall, these experiments indicate that a nitrite-NO pathway exists and is functioning in fruit flies. Based on this evidence, it is tempting to speculate that a backup system ensuring NO homeostasis is of enough physiologic importance to be selected by evolution and kept intact across species and time. It is fascinating also to note some similarities with the better characterized mammalian nitrate-nitrite-NO pathway [19]. The most striking is probably the involvement of bacteria in NO formation. An involvement that, according to the evidence presented in Figure 4B, might be nearly as important in *Drosophila* as in mammals (Study II) [170]. Expanding on this, the observation that bacterial cultures from flies become acidic while generating NO from nitrite, might suggest a strong contribution by *i.e.* lactic acid producing bacteria. One example is *Lactobacillus*, which might induce non-enzymatic and pH-driven reduction of nitrite by generating lactic acid. The excellent nitrite-reducing abilities of this bacterial genus have been reported in mammals [193] and we could indeed isolate *Lactobacillus* from *Drosophila* homogenates. It is also true that enzymatic reduction of nitrate/nitrite to NO is present in mammals [50]. Similarly, an additional contribution from the host to NO generation is suggested by this study in the fruit fly.

4.1.3 Dietary inorganic nitrite prevents age-related locomotor decline in female flies

When exploring interventions that positively affect the aging process, studying the healthspan may be of even greater relevance than investigating an organism´s lifespan. Therefore, we wanted to find out whether a longer life would be coupled to improved or delayed age-related functional decline upon nitrite consumption. Locomotor ability progressively deteriorates with aging in several species. In *Drosophila*, locomotion is a behaviour termed "negative geotaxis": an escape instinct whereby insects ascend the wall of a tube whenever they are knocked down to the bottom [162,194]. We performed a rapid iterative negative geotaxis (RING) assay to assess the locomotor behaviour of female flies from a younger age (7 days old) and during aging (30 and 44 days old). According to our findings, life-long nitrite supplementation (1 µM)

preserves the locomotor ability of aged (30 days old) female flies, as compared to untreated controls (Figure 5). This effect seems to persist in older flies (44 days), although not reaching statistical significance.

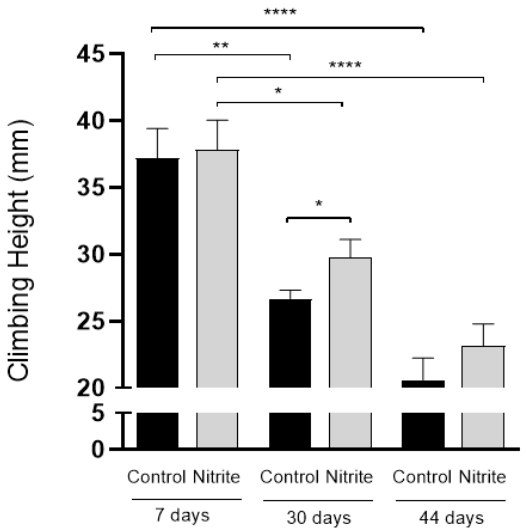

Figure 5. Height climbed by female flies subjected to a RING assay while fed a 15% SY media with or without 1 µM sodium nitrite ($NaNO_2$). Data are shown as mean ± SEM of two independent experiments. (n=6/group for each independent experiment). Mann-Whitney test was used for comparison within each timepoint and two-way ANOVA with Sidak correction for comparisons among different timepoints.

Other pro-longevity molecules such as resveratrol [195–198], are known to improve locomotion in flies and dietary nitrate itself is proving a promising intervention protecting physical function in older adults (as discussed in Chapter 1.7).
These results suggest that inorganic dietary nitrite, by promoting healthy aging, leads to life extension in the fly.

Improved mitochondrial efficiency is suggested to be involved in the exercise-enhancing effects of dietary nitrate in humans [13]. For this reason, we investigated mitochondrial function in isolated thorax muscle fibers of aged female flies supplemented with dietary nitrite (1 µM). As we could not detect any differences between the two groups, we anticipate that modulation of mitochondrial function might not explain the protective effect of nitrite on locomotor decline in the aged flies. One possible explanation could be species-specificity of the interaction between NO and the mitochondria. In support of this hypothesis, a recent study showed increases in voluntary running by nitrate in mice, but unlike in humans [13], the P/O ratio was decreased by nitrate [199]. Alternatively, it might be possible that the actions of nitrate and nitrite differ on mitochondrial function. As an example, in an *in vitro* setting, nitrite was previously reported to have no effect on mitochondrial LEAK or state 4 respiration from human muscle fibers [13].

4.1.4 Dietary inorganic nitrite modulates metabolic pathways in aged female flies

Our findings showing positive modulation of both the lifespan and healthspan by dietary nitrite, triggered our interest in exploring the potential molecular reasons behind such effects.

Nutrient sensing pathways are known to become dysregulated with aging as well as in metabolic disorders [200]. The insulin/IGF-1 pathway is an important modulator of the aging process and calorie restriction is known to extend longevity by suppression of this pathway. Other important nutrient sensors are AMPK and its downstream target TOR, as well as sirtuins. Both AMPK and sirtuins are downregulated by aging and their activation has been linked to increased longevity. Importantly, interventions modulating these pathways, do not exclusively extend lifespan but they also positively impact on metabolism by improving insulin sensitivity, glucose tolerance, physical performance and counteracting lipid accumulation as well as oxidative stress [201–203]. Most of these features have been observed following dietary nitrate supplementation [22,23], which is also known to activate AMPK [23].

We therefore assessed the mRNA levels of two important regulators of metabolism and aging, dTOR and d*Sir2* (homolog of the mammalian sirtuin SIRT1).
In line with our longevity studies, life-long nitrite supplementation (1 µM) reduced dTOR mRNA levels in aged female flies (Figure 6A; p=0.033). Downregulation of dTOR is known to mediate the longevity effect of DR and rapamycin [203]. Moreover, activation of AMPK also leads to TOR suppression and some AMPK-activators have been reported to extend lifespan [204,205]. One of these is metformin [204], which shares features with nitrate including the ability to activate AMPK [158].
In the same population of female flies, dietary nitrite led to a significant, although mild, upregulation of d*Sir2* mRNA levels (Figure 6B; p=0.036). In mammals, SIRT1 activators have gained much attention owing for their DR-mimetic effects on metabolism and aging [206]. Resveratrol is likely the most studied sirtuin activator of dietary origin. This polyphenol is known for its antioxidant properties, it has also been shown to protect mice from obesity, improve mitochondrial function, physical activity and extend lifespan [202].
Consistent with a sex-dependent effect of nitrite on the fly longevity, we did not detect significant changes in either dTOR or d*Sir2* in aged male flies (Figure 6C; D).

Supportive of a role for these nutrient sensing pathways in the modulation of the fly lifespan by nitrite, supplementation with this anion (1 µM) lowered total triglycerides and glucose levels in aged female flies.

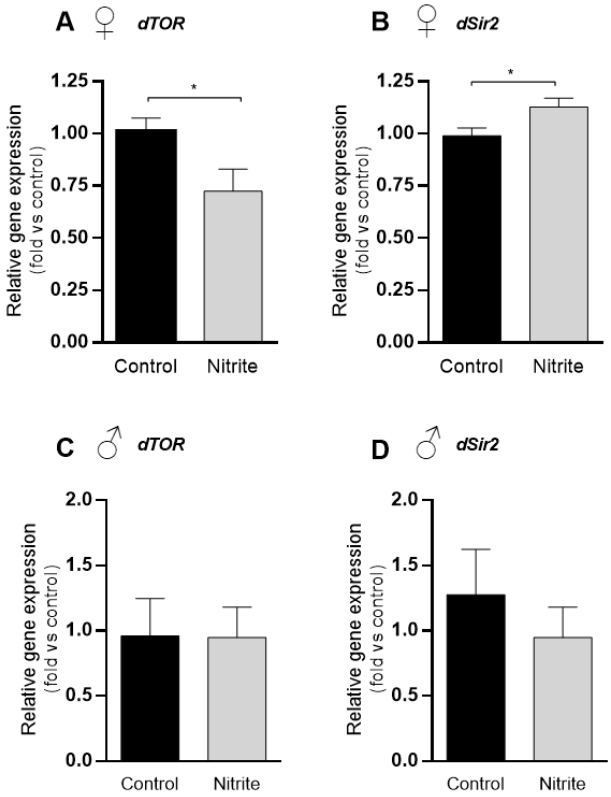

Figure 6. mRNA levels of dTOR (A; n=8) and *dSir2* (B; n=6) in 30 days old female flies and male flies (C; D respectively; n=6). Relative gene expression was calculated using the $2^{\wedge-\Delta\Delta Ct}$ method. Data are shown as mean ± SEM of each biologic replicate. Unpaired t-test was used to determine statistical significance (*$p < 0.05$).

Although indicative of nutrient sensing modulation by nitrite, our findings do not provide a causal link between the observed changes in dTOR and d*Sir2* mRNA levels and increased longevity. Future studies should address this matter, for instance by testing the effects of dietary nitrite in dTOR and d*Sir2* mutant flies supplemented with nitrite. Similarly, whether nitrite acts as a DR mimetic would need further clarification by studying the lifespan of fruit flies subjected to DR and nitrite supplementation simultaneously.

Finally, this study highlights the potential of boosting NO production via the diet as a mean to protect health during the aging process. Thus, it should be assessed if the pro-longevity effects reported here could be reproduced by dietary nitrate in mammals.

4.2 STUDY II

The obligatory role of host microbiota in bioactivation of dietary nitrate

4.2.1 Effects of a Western diet and dietary nitrate on the body weight of germ-free and conventional mice

The pivotal role of our bacteria in bioactivating dietary nitrate, ensuring its beneficial biologic effects have been described in Chapters 1.3 and 1.4. In addition to this established knowledge,

in Study I we revealed that even in the fruit fly, a surprisingly important relationship between bacteria and their host drives NO generation from dietary nitrite.

In this study, we used GF mice to clarify the importance of host microbiota in the cardiovascular and metabolic effects of dietary nitrate-derived NO bioactivity. To do so, we induced metabolic dysfunction and hypertension in both GF and CONV mice by feeding them a combination of a Western diet and the NOS inhibitor L-NAME (1 g/L in drinking water) for 6 weeks. We supplemented half of these mice with sodium nitrate ($NaNO_3$, 10 mM) or placebo (NaCl, 10 mM) in drinking water and compared them to a group of untreated mice (control).

At the end of the dietary challenge, both GF and CONV mice had developed an obese phenotype, with increased fat mass (Figure 7C; D) and decreased lean mass (Figure 7E; F). Nonetheless, CONV mice in the nitrate group gained weight to a lesser extent than the placebo group, as compared to untreated CONV mice (Figure 7A). Accordingly, the increase in fat mass was milder in CONV mice supplemented with nitrate than in the CONV placebo group ($p=0.056$; Figure 7C). No such trend was observed in GF mice, whereby a Western diet significantly increased body weight (Figure 7B) and fat mass (Figure 7D), irrespective of the nitrate treatment.

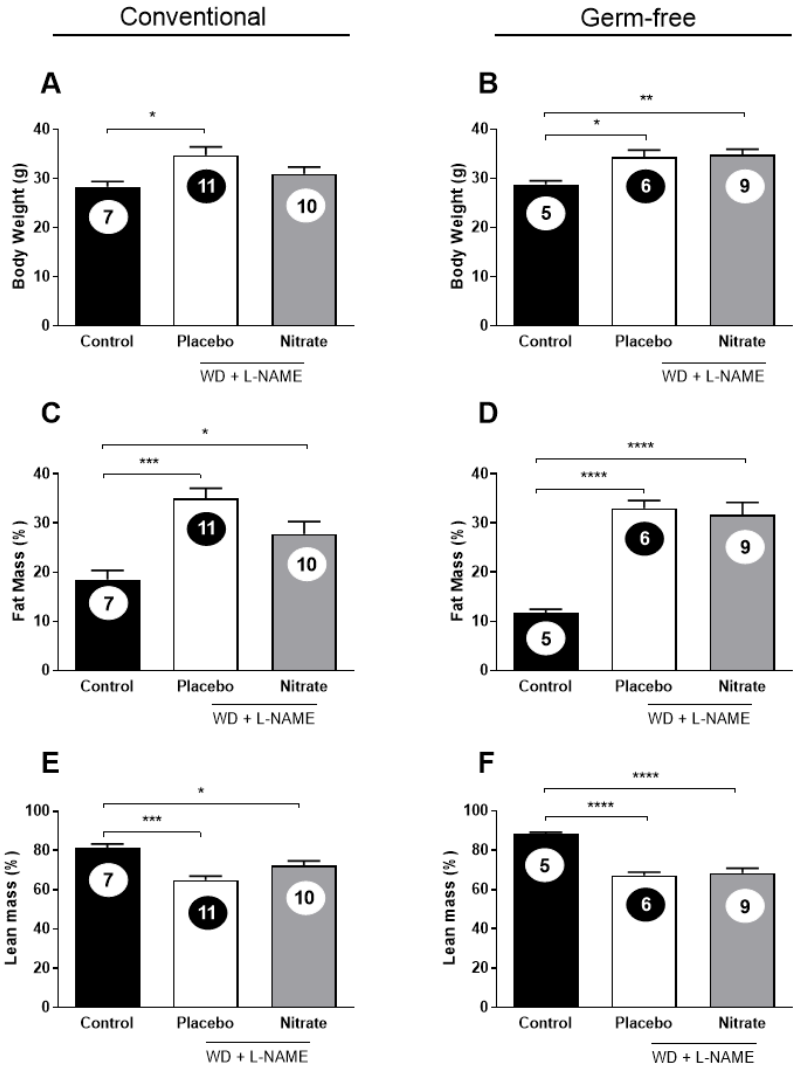

Figure 7. Body weight (A; B), fat (C; D) and lean mass (E; F) of CONV and GF mice following 6 weeks on a standard rodent diet (control) or Western diet (WD) + L-NAME (1 g/L), with either 10 mM NaCl or 10 mM $NaNO_3$ in the drinking water. Data are shown as mean ± SEM. One-way ANOVA with Tukey post-test was used for multiple comparisons. n numbers aa indicated within the bar chart.

The salutary effects of dietary nitrate have been described in several animal models in respect to metabolic function and, to a greater extent, cardiovascular function [23,27]. However, while the anti-hypertensive and anti-diabetic actions of inorganic nitrate have been predominantly consistent among studies, decreased body weight or prevention of weight gain by dietary nitrate have been indicated in some reports [80,87,93,207,208] but not in others [158,169,209]. Thus, the potential benefits of inorganic nitrate as an anti-obesity agent need further clarification [210]. Nonetheless, based on the data presented above, a trend towards a protective effect of nitrate on diet-induced weight gain in CONV mice is totally lacking in GF mice. This indicates that even enhancing a nitrate-driven weight loss would require bacterial bioactivation of nitrate.

4.2.2 The antidiabetic effects of dietary nitrate are dependent on the microbiota

In CONV mice but not in GF mice, dietary nitrate effectively prevents the deleterious effects on glucose tolerance caused by a protracted Western diet consumption (Figure 8).

Accordingly, Cordero et al. (Study III) reported no effects of nitrate supplementation on diet-induced hepatic steatosis in GF mice. On the contrary, such complication of T2D was effectively prevented by nitrate in CONV mice [209]. Together, these findings provide new and strong evidence on the obligatory role of the host microbiota in the antidiabetic effects of inorganic nitrate.

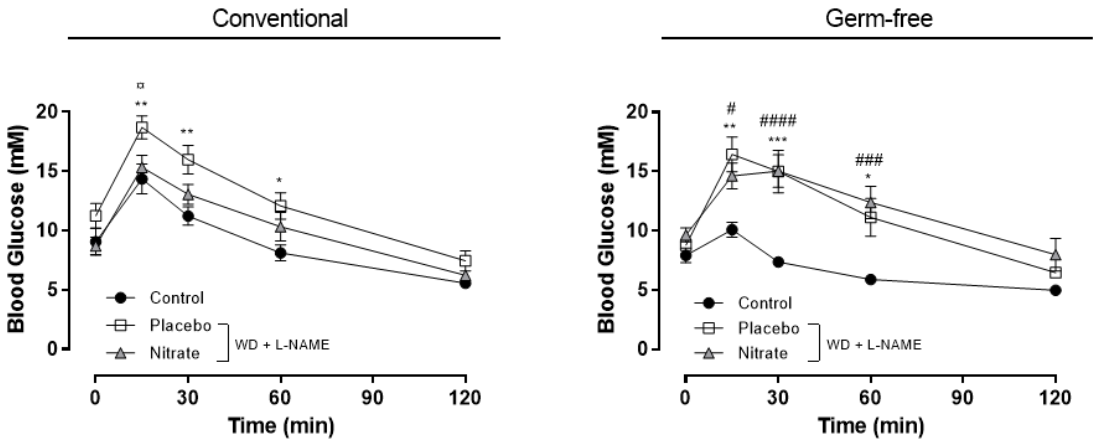

Figure 8. Glucose tolerance tests in CONV and GF mice after 6 weeks on a standard rodent diet (control; n=5) or a Western diet (WD) + L-NAME (1 g/L) with either 10 mM NaCl (n=6) or 10 mM $NaNO_3$ (n=9) in the drinking water. Data are shown as mean ± SEM Two-way ANOVA with Tukey post-test was used for multiple comparisons. * placebo vs control; # nitrate vs control; ¤ placebo vs nitrate.

4.2.3 The cardiovascular effects of dietary nitrate are dependent on the microbiota

Six weeks on a Western diet and L-NAME produced an increase in mean arterial blood pressure (MAP) in both GF and CONV mice, as compared to their respective control groups. However, CONV mice supplemented with dietary nitrate were protected from such increase. This effect was not achieved in the absence of bacteria in the GF animals (Figure 9).

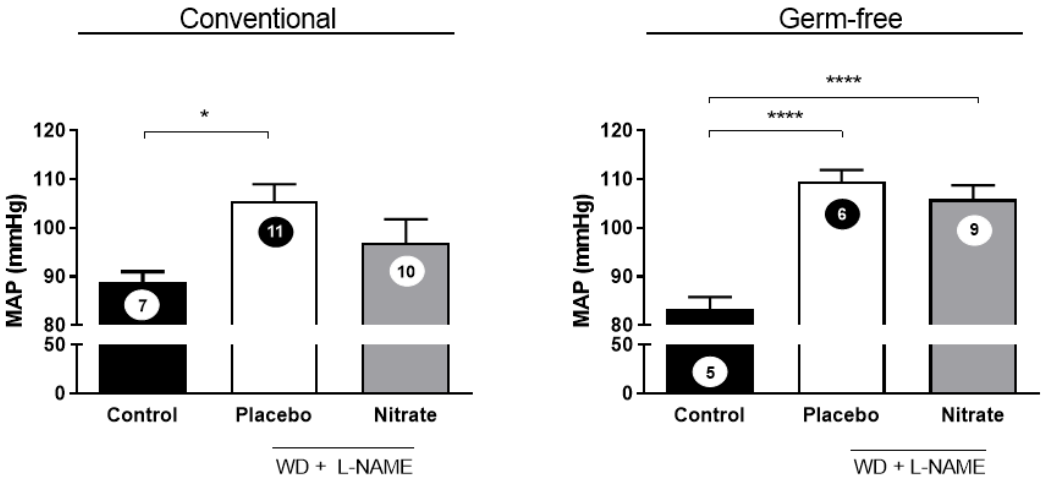

Figure 9. Mean arterial blood pressure (MAP) of CONV and GF mice after 6 weeks on a standard rodent diet (control) or a Western diet (WD) + L-NAME (1 g/L) with either 10 mM NaCl or 10 mM $NaNO_3$ in the drinking water. Data are shown as mean ± SEM One-way ANOVA with Tukey post-test was used for multiple comparisons.

The nitrate dose used in this study (10 mM) was previously shown to elicit clear blood pressure and gluco-regulatory effects when administered orally in rodents [30]. This dose is higher compared to what is normally used in human studies but takes into account the demonstrated lower ability of rodents to concentrate nitrate in salivary glands, as compared to humans [211]. Nevertheless, we cannot exclude that a higher dose would trigger an anti-hypertensive response even in GF mice. To this regard, an earlier study by Jansson et al. [50] reported lowered blood pressure by intravenously administered nitrate in CONV anesthetized rats. This led the authors to anticipate a mammalian enzymatic contribution to the bioactivation of nitrate and its effects on blood pressure. However, in these animals the swallowing reflex was likely inhibited by the anaesthesia, thus disrupting the enterosalivary nitrate circulation. Moreover, according to recent studies [212], it is possible that gut bacteria instead of oral bacteria were responsible for the nitrate reduction to nitrite in the Jansson study. In fact, bacterial reduction of circulating nitrate can also take place in the small intestine, leading to increased nitrite levels in the portal circulation [212].

Importantly, our findings do not exclude that mammalian enzymes, such as XOR as indicated by Jansson et al. [50], might play an important role in the nitrate-nitrite-NO pathway. This could be especially true under certain conditions such as hypoxia, when NO formation from nitrate/nitrite is enhanced [171,213] or within specific tissues where the high expression of XOR prompts mammalian nitrate bioactivation [114].

4.2.4 The NO signaling downstream of nitrate is intact and functioning in germ-free mice

To validate the idea that GF mice do respond to an exogenous stimulation of NO production, we performed an acute *in vivo* experiment whereby GF and CONV mice were given a high dose (15 mg/kg body weight) of sodium nitrite ($NaNO_2$) orally. Following this procedure, GF

and CONV mice both showed decreased MAP (Figure 10). Finally, to further strengthen this evidence, additional *ex vivo* evaluation of vessel response showed equal vasorelaxation of GF and CONV mesenteric arteries to the NO donor sodium nitroprusside (SNP).

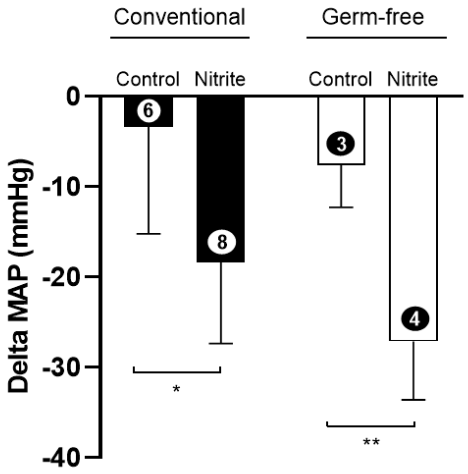

Figure 10. Changes in mean arterial pressure (Delta MAP) of CONV and GF mice after oral administration of either vehicle (control) or sodium nitrite ($NaNO_2$; 15 mg/kg of body weight). Data are shown as mean ± SEM. Statistical significance was assessed by unpaired *t*-test.

Together, these experiments confirm that in GF mice, the ability to reduce nitrite to NO as well as the physiologic response to NO are both intact. Thus, a lack of a biological effect in response to nitrate supplementation in the GF mouse is attributed to the complete absence of bacteria and their ability to generate of nitrite.

4.3 STUDY III

AMP-activated protein kinase activation and NADPH oxidase inhibition by inorganic nitrate and nitrite prevent liver steatosis

4.3.1 Dietary nitrate protects against hepatic steatosis: the indispensable role of bacteria

The aim of this study was to broaden our knowledge on the protective effects of dietary nitrate in T2D and hepatic steatosis, when these are secondary to obesity. Similar to Study II, a mouse model of metabolic disease and hypertension was created by high fat (HF) diet feeding coupled with NOS inhibition (L-NAME, 1 g/L in drinking water) for 7 weeks. In line with previous reports [23,27], supplementation with inorganic nitrate (1.0 mmol kg of body weight per day) prevented a L-NAME-driven elevation in blood pressure as well as glucose intolerance and endothelial dysfunction.

Mice fed a HF diet showed hepatic lipid accumulation, which was prevented by dietary nitrate (Figure 11A). However, when the same *in vivo* model was recreated in the GF mouse, these protective effects of dietary nitrate could not be reproduced (Figure 11B). This evidence, together with the findings reported in Study II, further strengthens the idea of an obligatory role

of the microbiota in bioactivation of dietary nitrate, which is indispensable for its metabolic effects.

To better explore the mechanisms behind a NO-driven protection against hepatic steatosis, we created an *in vitro* model of the disease. Human hepatocyte cell line HepG2 were incubated for 24h with glucose, insulin and a free fatty acids (FFA) mixture. Staining of these cells with Oil Red O, confirmed the accumulation of lipids. In line with the *in vivo* study, incubation of the cells with nitrite prevented such effect. Importantly, these results were obtained using sub-micromolar concentrations of nitrite, which are close to the physiologic levels in tissues and easily achievable via the diet [19,214].

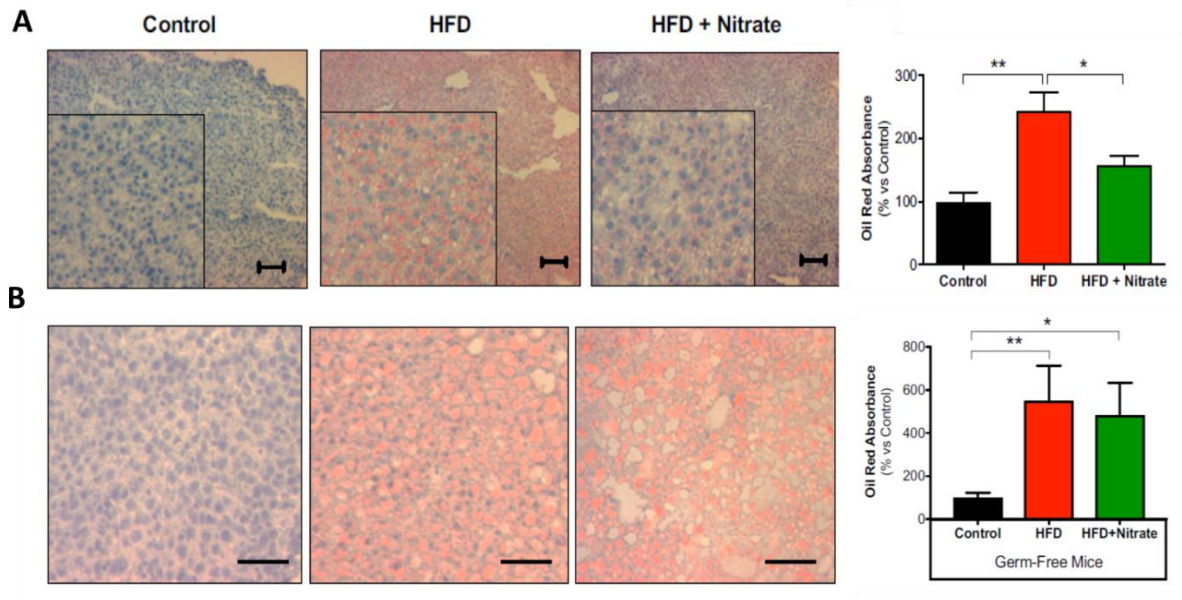

Figure 11. Oil-Red O staining of hepatic tissue of CONV (A) and GF mice (B) after 7 weeks on a standard rodent diet (control) or a HF diet with L-NAME (1 g/L) and either sodium nitrate ($NaNO_3$; 1.0 $mmol \cdot kg^{-1} \cdot d^{-1}$) or placebo (NaCl) in drinking water (scale bars: 1 μm). Quantification of neutral lipids was performed using ImageJ software. Data are presented as mean ± SEM of n = 5-9 per group. Statistical significance was determined by Kruskal–Wallis test and Dunn's test. *Modified from 10.1073/pnas.1809406115.*

4.3.2 Nitrate and nitrite activate AMPK and reduce NOX activity

AMPK is a master regulator of glucose and lipid metabolism, playing a key role in hepatic lipid accumulation [215] and excess of nutrients is known to suppress its activation [102,103,216]. Accordingly, mice fed a HF diet displayed decreased levels of p-AMPK (active form) as compared to untreated controls. When the HF diet was paired with nitrate supplementation, such effect was prevented (Figure 12A). These same results were reproduced in the *in vitro* model of hepatic steatosis described above.

The PI3/Akt signaling pathway is triggered by insulin and plays a crucial role in T2D and obesity [217]. According to our results both in liver tissue and cells, Akt does not seem to be involved in the anti-steatotic effects of nitrate and nitrite (Figure 12B). Instead, in line with

previous reports [218], we indicate that these are mediated by insulin-independent activation of AMPK.

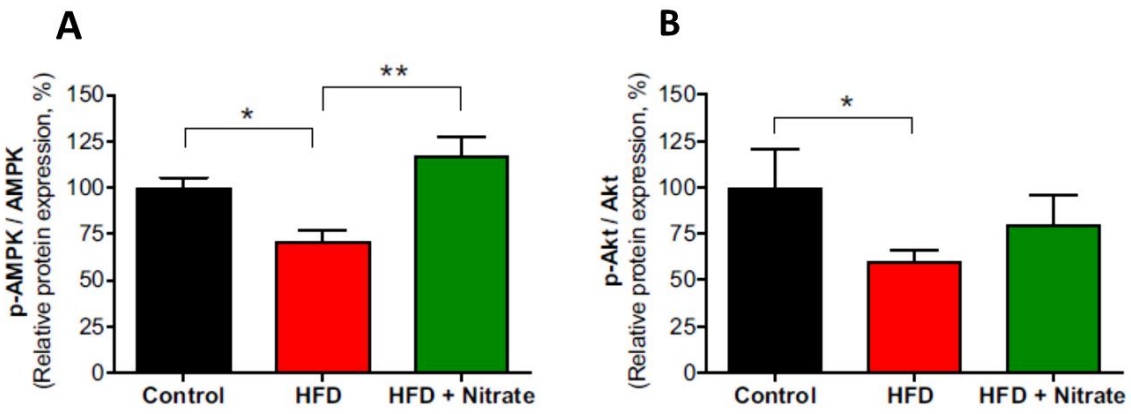

Figure 12. Protein expression of AMPK (A) and Akt (B) in hepatic tissue of mice after 7 weeks on a standard rodent diet (control) or a HF diet with L-NAME (1 g/L) and either sodium nitrate ($NaNO_3$; 1.0 $mmol \cdot kg^{-1} \cdot d^{-1}$) or placebo (NaCl) in drinking water. Data are presented the mean ± SEM of n= 9-10 per group. Statistical significance was determined by Kruskal–Wallis test and Dunn's test. *Modified from 10.1073/pnas.1809406115.*

Dietary nitrate supplementation is known to reduce NOX activity and the resulting antioxidant effect has been previously associated with modulation of metabolic function by nitrate [22,108]. In our *in vivo* model of metabolic disease, chronic HF feeding increased hepatic NOX activity and NOX-driven superoxide production. A positive correlation between NOX activity and lipid accumulation in the liver was also noted. On the other hand, when mice were supplemented with nitrate, the increase in hepatic NOX activity was prevented.
Similarly, in the *in vitro* model of hepatic steatosis, incubation with the steatotic mixture elevated NOX-derived superoxide production and this effect was absent when cells were exposed to nitrite. Confirming the role of NOX-derived superoxide generation in lipid accumulation, cells were incubated with either the superoxide scavenger tempol or with two different NOX inhibitors. In both cases lipid accumulation was prevented.

4.3.3 NO is responsible for the anti-steatotic effects of nitrate and nitrite

Nitrite exerts its biologic effects following a bioactivation process, not exclusively to form NO but also other bioactive nitrogen oxides such as S-nitrosothiols and the nitrogen dioxide radical [219]. Therefore, we sought to verify whether the effects seen *in vitro* following exposure to nitrite would be mediated by NO. To do so, we tested if increased or decreased NO signaling would modulate AMPK and NOX activity in accordance with the previously obtained results.

In steatotic HepG2 cells incubated with the NO donor DETA-NONOate or the cGMP activator 8p-CPT-cGMP, we obtained similar effects on AMPK as with nitrite *in vitro*. This indicates that the biologically active molecule responsible for modulation of AMPK signalling, and ultimately the anti-steatotic effects, is in fact NO. Similarly, NOX inhibition was mimicked by

both DETA-NONOate and 8-pCPT-cGMP and abolished by the sGC inhibitor ODQ in the presence of nitrite.

XOR and aldehyde oxidase (AO) are two molybdopterin-containing enzymes known to catalyse the reduction of nitrite to NO [220]. To investigate their involvement in nitrite reduction to NO in the *in vitro* setting, the XOR inhibitor febuxostat and the AO inhibitor raloxifene were used. XOR inhibition blunted the positive effects of nitrite on NOX activity and lipid accumulation. On the contrary, raloxifene did not affect the benefits of nitrite treatment on either NOX or lipid deposition. Moreover, when NO generation from nitrite was detected by electron paramagnetic resonance (EPR) spin trapping, febuxostat attenuated but did not fully abolish a previously more prominent NO signal. This evidence suggests a role for XOR in bioactivation of nitrite but also indicates that other nitrite-reducing enzymes [213] may be at work in this *in vitro* model.

In summary, the findings in Study III bring to light a remarkable role of inorganic nitrate and nitrite in regulating glucose and lipid metabolism. We also show that these effects are driven by NO and therefore, bacterial bioactivation of dietary nitrate is required. Finally, activation of the metabolic regulator AMPK and inhibition of NADPH oxidase are suggested as some of the molecular drivers in the described protective effects of nitrate and nitrite.

4.4 STUDY IV

Germ-free mice are not protected against diet-induced obesity and metabolic dysfunction

4.4.1 Germ-free mice develop an obese phenotype when fed a Western diet

If the oral microbiota represents an fundamental player in the biologic effects of nitrate-derived NO, the microbes populating our gut are believed indispensable to harvest energy from the diet and to direct the accumulation of dietary fats [53,54].

However, in Study II of this thesis GF and CONV mice gain weight to a similar extent following sustained (6 weeks) consumption of a calorie dense Western diet. This specific finding triggered our curiosity, as earlier studies had shown obesity resistance of GF mice [53–55]. However, when revising the literature in search for explanations to our observations, we found that a few others had reported weight gain in GF mice [62–64]. For these reasons, we decided to further investigate whether the microbiota is indeed required for fat storage, hence the development of obesity. To do so, we used the same mouse strain and Western diet as in the original study [53] reporting obesity resistance in GF mice.

Thus, aged-matched GF and CONV mice received a Western diet for 16 weeks and body weight, food and water consumption were monitored weekly. During this time, both groups of mice progressively gained weight to a similar extent (Figure 13A). By the end of the dietary intervention, GF mice had gained 13 ± 1.5 g and CONV mice 14.7 ± 1.7 g from baseline (Figure

13B). These observations were confirmed by body composition analyses (Figure 13C) and occurred despite similar food and water intake in the GF and CONV groups (Figure 13D).

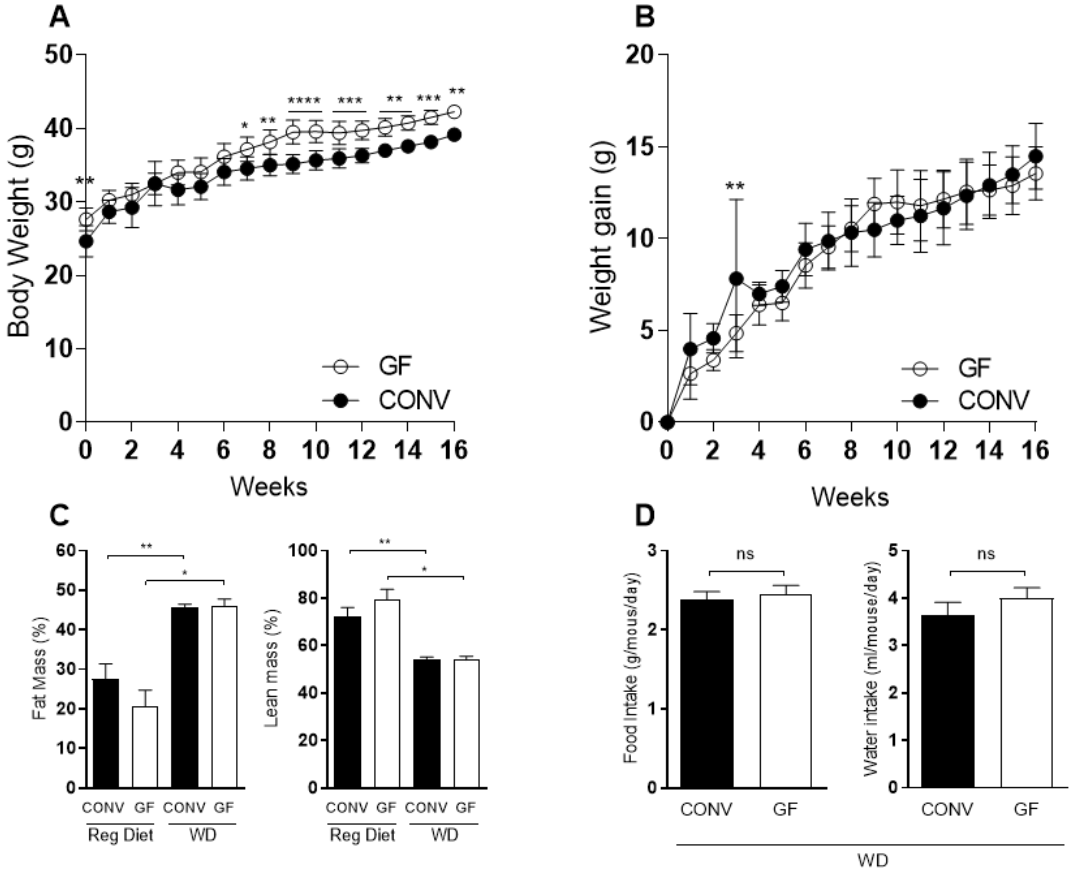

Figure 13. Body weight (A) and body weight gain from baseline (B) of CONV (n=6) and GF mice (n=10) during 16 weeks on a Western diet (WD). Body composition (C) and cumulative food and water intake (D) of GF (n=3) and CONV mice (n=4) fed a regular rodent diet or a WD for 16 weeks. Data are shown as mean ± SD (A; B) or ± SEM (C; D). *p* values were calculated with 2-way ANOVA (A; B). Comparisons CONV + Reg Diet vs CONV + WD; GF + Reg Diet vs GF+WD; CONV + WD vs GF + WD were analysed by Mann-Whitney test (C) or *t*-test (D).

The reason why GF mice are protected from obesity in some studies [53–55] but not in the current and some other studies [62–65] appears unclear. Differences in experimental design, mouse strain and diets are so far the prevalent hypotheses to explain discrepancies among reports [64]. We herein attempted to mimic the original study reporting obesity resistance in GF mice [53] by using the same diet and the same mouse strain. Yet, our GF mice became obese upon prolonged Western diet consumption. Nonetheless, we should not forget that the host-microbiota relationship is still somewhat mysterious, and these discordant findings might hide new and interesting information. For instance, GF mice in the first study showed increased locomotor activity, as compared to CONV mice [53]. At that time, in 2007, very little was known about a microbiota-gut-brain axis. Although our knowledge is still limited, we know today that the microbiota modulates normal stress responsivity, anxiety-like behaviours, cognition and even social behaviour [221]. Thus, any environmental factor likely affecting

behaviour (and potentially food intake, physical activity and social interactions as a consequence), might represent one of the causes for the different outcomes obtained in GF mice bred in different facilities. Moreover, even using the same mouse strain, differences in genetic background are not to be excluded.

4.4.2 Germ-free mice on a Western diet develop impaired glucose metabolism

In light of the findings above, we decided to study glucose tolerance in our GF and CONV mice. Intake of a Western diet increased fasting blood glucose in both groups (Figure 14A) and a glucose tolerance test revealed a compromised response to glucose in both GF and CONV mice in the Western Diet groups, as compared to untreated controls (Figure 14B; C).

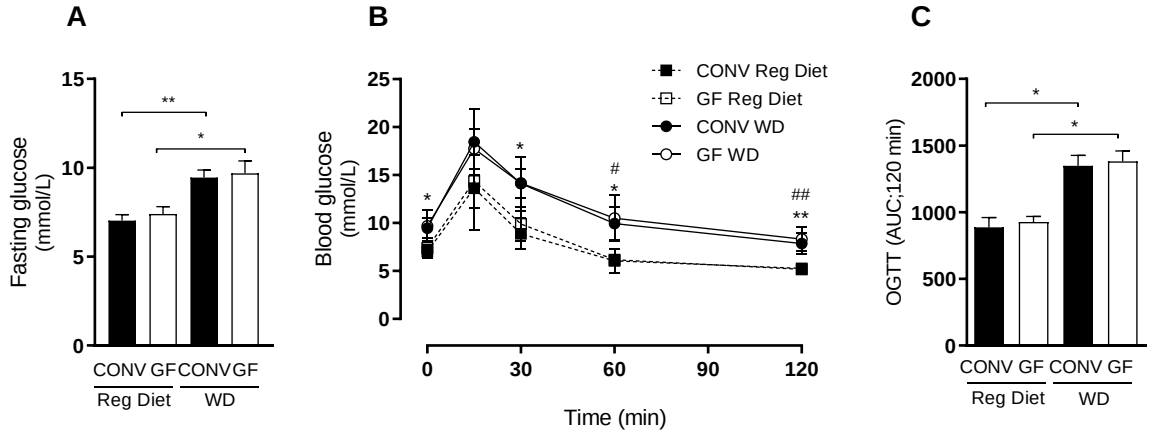

Figure 14. Fasting blood glucose (A) glucose tolerance test curve (B) and area under the curve (C) of CONV (n=4) and GF mice (n=3) fed a regular rodent diet or a Western diet (WD; GF n=6 and CONV n=6) for 16 weeks. Data are shown as mean ± SEM. Comparisons are CONV + Reg Diet vs CONV + WD; GF + Reg Diet vs GF+WD; CONV + WD vs GF + WD, analysed with two-way ANOVA (B) or Mann-Whitney test (A; C). * CONV + Reg Diet vs CONV + WD; # GF + Reg Diet vs GF+WD

4.4.3 Germ-free mice on a Western diet develop liver steatosis

Consistent with the idea that the absence of a microbiota prevents body weight gain and its related metabolic impairments, GF mice in previous studies were also shown to be protected from liver steatosis [222]. However, from a macroscopic examination of the livers, GF mice displayed a clear ectopic lipid accumulation (Figure 15E). When assessing hepatic lipid accumulation on a microscopic level by Oil Red O staining, we found that both GF and CONV mice fed a Western diet had developed a steatotic-like phenotype (Figure 15A; B). This was confirmed by quantification of hepatic triglycerides (Figure 15D).

Accordingly, the weights of the livers were significantly increased in the animals receiving a Western diet, as compared to their untreated counterparts (Figure 15C).

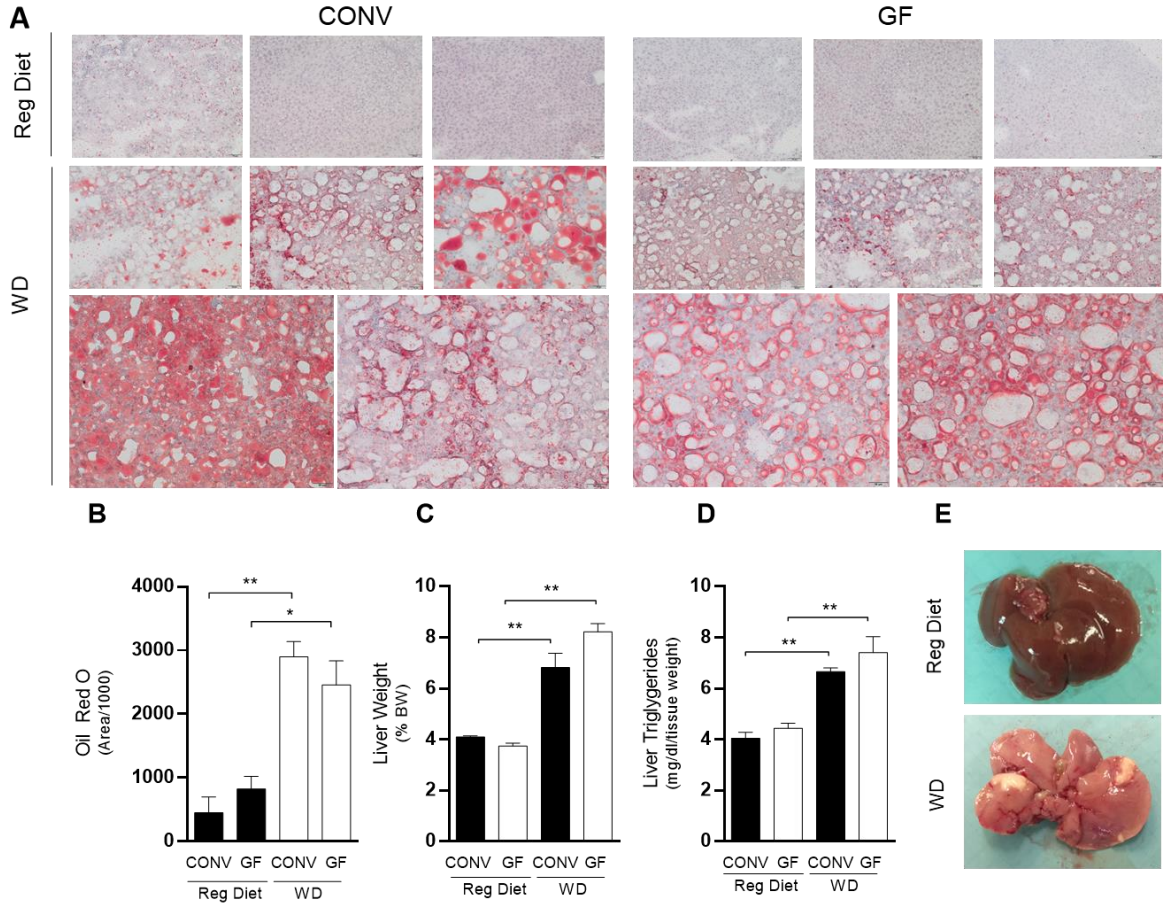

Figure 15. Representative pictures of hepatic tissue stained with Oil Red O (A); quantification of Oil Red O stained area (B); liver weight (C); liver triglycerides (D) and representative pictures of livers (E) of CONV (n=4) and GF mice (n=3) fed a regular diet or a Western diet (WD; CONV n=6 and GF n=6) for 16 weeks. Data are shown as mean ± SEM. Comparisons CONV + Reg Diet vs CONV + WD; GF + Reg Diet vs GF+WD; CONV + WD vs GF + WD were analysed with Mann-Whitney test.

Altogether, we present evidence that the host microbiota is not an indispensable factor for lipid accumulation and weight gain. Thus, in otherwise healthy individuals, this is likely primarily driven by excessive energy intake and consequently energy imbalance.

4.4.4 CPT1 activity is elevated in skeletal muscle of obese germ-free mice

Carnitine palmitoyl transferase 1 (CPT1) is the rate limiting enzyme for mitochondrial fatty acid oxidation and its activity was previously found to be elevated in GF mice as compared to colonized mice fed a Western diet [53]. This led to the hypothesis that the microbiota might suppress CPT1 activity and thereby promote fat storage. Surprisingly, elevated CPT1 activity was observed also in our GF mice fed a Western diet as compared to CONV mice on the same diet (p=0.008). However, in our case this was not associated with resistance to diet-induced obesity.

The role of fatty acid oxidation in diabetes and obesity appears somewhat unclear. Some studies across the literature support the idea that inhibition of fatty acid oxidation might ameliorate insulin resistance [223,224] whereas others found its stimulation to elicit the same beneficial effects in rodents [225]. Future research should clarify the role of human fatty acid oxidation in metabolic diseases.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

After the discovery of the nitrate-nitrite-NO pathway in 1994, inorganic nitrate gained increasing attention as a dietary means of generating NO bioactivity. Owing to the known vasodilatory properties of this small gaseous molecule, researchers immediately started to explore the therapeutic value of nitrate supplementation to restore NO homeostasis in cardiovascular disease [19,22,226]. In 1995, it was reported that $eNOS^{-/-}$ mice not only develop hypertension but also severe metabolic dysfunction [82]. These findings revealed a physiologic role of NO that goes beyond the regulation of blood pressure. Indeed, later pioneering studies by Larsen and colleagues, proved that boosting NO production via nitrate supplementation enhances metabolic efficiency in humans by decreasing resting metabolic rate [227] but also oxygen cost during exercise [117], while enhancing mitochondrial efficiency in healthy individuals [13].

Thus, dietary nitrate was emerging as a modulator of metabolic function, holding promise as a dietary intervention that promotes and protects overall good health. This idea triggered our curiosity about any anti-aging potential of dietary nitrate. Notably, NO synthesis and bioavailability decrease during aging [7], when cardiovascular, metabolic and physical function decline as well.

When this work was started in 2016, our group had just shown beneficial effects of nitrate supplementation on blood pressure, endothelial function and glucose homeostasis in a rodent model of natural aging [74]. However, a straightforward answer on whether diet-derived NO could extend health- and lifespan, would only come from longevity studies.
The tiny insect *Drosophila melanogaster* enabled us to show that low amounts of inorganic dietary nitrite exert a pro-longevity effect in female flies and protect them from age-related locomotor decline. In the same study, we indicate that fruit flies effectively reduce nitrite, forming NO. In insects like in other species, NO is a key signaling molecule essential for development [228] and a variety of life-indispensable processes [229]. Thus, based on our findings, it is tempting to speculate that backup NO generation from exogenous nitrite and nitrate might represent an evolutionary preserved mechanism, reinforcing the idea of NO as a crucial molecule for life. What is more to this, is the extraordinary contribution of the fly commensal bacteria to NO formation from nitrite.

The obligatory role of the mammalian microbiota in bioactivation of dietary nitrate is another key finding of this thesis. Using germ-free mice, we unequivocally show that bacteria represent the *sine qua non* for both the cardiovascular and metabolic benefits of dietary nitrate.

Together, these studies bring to light an ancient story of generosity between the host and its microbiota, whereby bacteria are provided with important substrates (nitrate and nitrite) for their own respiration and, in a true symbiotic fashion, give back to the host a tiny gaseous molecule crucial for life-long health: nitric oxide.

Further, we add new knowledge on the potential of dietary nitrate in preserving metabolic health, both when this is threatened by unhealthy dietary habits but also during the natural process of aging. To this regard, one interesting observation in this thesis is the sexual dimorphism in the pro-longevity effects of nitrite. In 2018, Kapil and colleagues reported increased nitrate reduction activity in females, both at baseline and after nitrate ingestion. This suggests that the oral microbiota in females might be a more efficient nitrate reducer. Nonetheless, the oral microbiota did not seem to differ between sexes in the Kapil study [230], although the scientific community tends to agree on sex-differences in microbial composition [231,232]. Clarifying whether a sexual dimorphism in nitrate reductase capacity is to be attributed partly or completely to the oral flora might provide useful information on how to maximize the benefits of nitrate supplementation. For instance, specific probiotic interventions could be optimized to ensure efficient nitrate bioactivation in otherwise non-responder subjects. These might also be of value for older adults, patients with specific oral diseases or subjects chronically treated with medications that could partly deplete or modify the oral microbiota.

In the past two decades, the gut microbiota has emerged as a crucial regulator of our metabolism and a key player in the onset of obesity and T2D [233]. However, in Study IV we provide evidence that calorie dense diets likely remain the main drivers of such metabolic disorders. Vegetable and fruit-rich diets - thus nitrate-rich diets - are known for their broad health benefits [71] and salutary impact on the gut microbiota [73,234]. Consequently, one would expect that, if any, the action of inorganic nitrate and nitrite in dietary amounts would positively shape gut bacteria and favour metabolic health in humans. Indeed, recent studies have already suggested a previously unbelieved interaction between the gut microbiota and inorganic nitrate, apparently with interesting implications in the salutary effects of this anion [78,80,81,235].

Whether or not dietary nitrate directly shapes the gut microbiota, one exciting opportunity would be to investigate whether this anion could indirectly produce such effect. Indeed, physical exercise has been shown to positively impact on the gut microbiota [236], with beneficial implications both in sports performance [237] and general health [236]. A possibility could be that nitrate supplementation, via its established exercise-enhancing abilities, might shape and maintain a "healthy gut microbiota" providing further protection against metabolic dysfunction. This information, would gain further clinical relevance if we hypothesize that

nitrate intake paired with exercise, might synergize in regulating blood glucose levels in diabetic patients.

In summary, the studies in this thesis bring to light novel and beneficial metabolic effects of inorganic nitrate and nitrite, two anions that have long been regarded as detrimental for our health. Importantly, this work also highlights the idea that protection of metabolic homeostasis by diet-derived NO involves various organ systems, eventually leading to overall promotion of health and perhaps even extension of the healthspan. Finally, our work shows that commensal bacteria play a key role in mediating the health benefits of dietary nitrate. Although, surprisingly, we found no support for the reigning dogma stating that gut bacteria drive diet-induced obesity in general.

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My PhD has been an amazing journey and what I learned goes well beyond science. I learned that failure is not a good reason to give up if you strongly believe in something. I learned to translate *unclear, negative* (result) to *interesting*. I learned that failures and "interesting" results are instead good reasons to research.

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